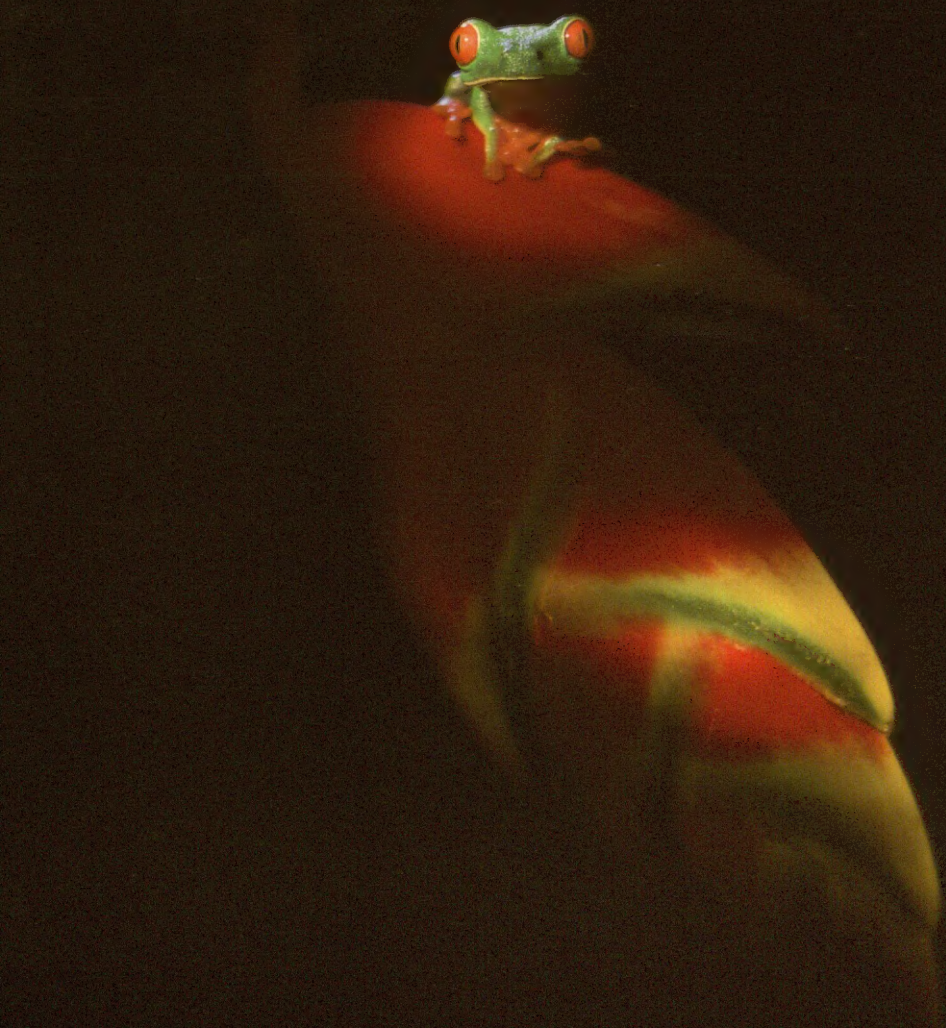
Realizing Nature's Potential

Proceedings of the
William L. Brown Symposium Honoring

Dr. Gordon Cragg



Bruce E. Ponman and James S. Miller, editors

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Missouri Botanical Garden

Missouri Botanical
Garden Library

ISBN 978-1-930723-96-2
Library of Congress Control Number 2010922967

Monographs in Systematic Botany from the Missouri Botanical Garden, Volume 118
ISSN 0161-1542

Scientific Editor and Head: Victoria C. Hollowell
Managing Editor: Beth Parada
Associate Editor: Allison Brock
Associate Editor: Tammy M. Charron
Press Coordinator: Cirri R. Moran

Design and typesetting by Bruce E. Ponman, William L. Brown Center at the Missouri Botanical Garden

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P.O. Box 299
St. Louis, Missouri 63166-0299, U.S.A.
www.mbgpress.info

Copyright is not claimed on *Marine-derived Compounds: New Frontiers in Production*, by David J. Newman, or on *Transdisciplinary Team Science and Lessons from the International Cooperative Biodiversity Groups*, by Flora N. Katz and Joshua P. Rosenthal, as the authors produced the work as employees of the United States government.

Printed in the U.S.A.

Cover photo: ©Gregory Basco/www.deepgreenphotography.com

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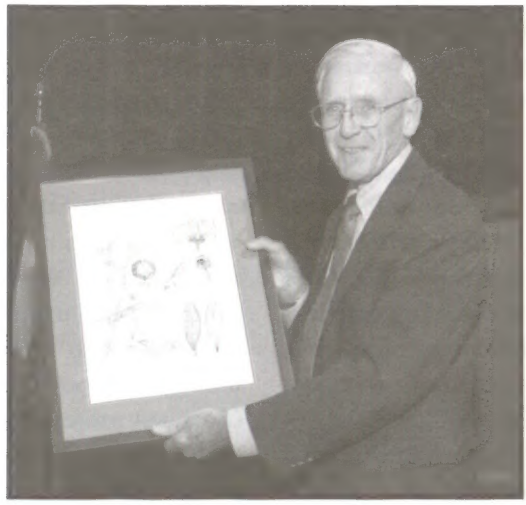

A Tribute to Gordon Cragg

James S. Miller

Throughout the course of history, nature has provided remedies for the diseases and other health issues we face. In early societies, and even in traditional cultures today, simple plant-based remedies are a source of primary health care. And in our modern world perhaps a third of pharmaceutical ingredients come from plants, animals, or microbes, and an even greater percentage owe their origin in some way to the study of natural products chemistry.

No one understands the value of nature to the drug discovery process better than Gordon Cragg. He has devoted his professional life to supporting and conducting natural products discovery research, but his reach has gone far beyond his own role as a research scientist. He has been an outspoken supporter of natural products discovery, he has encouraged other researchers in the field from students to senior research scientists, he has helped foster international collaboration, and he has been at the center of efforts to ensure that the contribution of raw materials from biodiverse tropical countries is recognized and that those countries equitably share in the research process and any of its achievements. As interest in natural products in the pharmaceutical industry has waxed and waned through several cycles, Gordon has been a vocal proponent of the value of plants, marine invertebrates, and other organisms as a source of novel chemistry with bioactive compounds of great complexity beyond what would likely be produced in synthetic research. He has been a major voice in support of the continuing and active interest in natural products at the National Institutes of Health. Gordon always has time and encouragement for everyone in the field, and whether they are among the most accomplished scientists or brand new students, he has always been generous with his time and willing to help make connections to support their work.

One of Gordon's greatest contributions has been his efforts to develop an ethical framework for bioprospecting that recognizes the true value of the biological resources that are surveyed, involves international scientists from source countries in meaningful ways, develops research capacity in source countries, and ensures that source countries receive an equitable share of any benefits, be they monetary or non-monetary, that arise

from these research efforts. My own experience began with Gordon when we were working to obtain permission to collect plant samples for the National Cancer Institute (NCI) in Madagascar and my colleague Dr. Rabodo Andriantsiferana asked "How will Madagascar benefit from this research?" This was in 1989, three years before the Convention on Biological Diversity even opened for signature, but it led to the kind of discussion that the Convention later aimed to promote, and a landmark agreement was signed between the government of Madagascar and NCI in 1990. Gordon had the patience and put in the work needed to fully understand the Malagasy position, and he worked and lobbied to push NCI to acknowledge that the biological materials from Madagascar were an essential component of the research process and that Madagascar deserved to share in potential revenues every bit as much as those contributing intellectual property in the United States. To date, NCI's original Letter of Intent, now Letter of Collection, remains a model document that has not only led to more ethical bioprospecting, but led many to reexamine their relationships with international collaborators and to establish much fairer partnerships.

Gordon and his colleagues also realized that the potential financial revenue from the discovery of a new drug would be only one way in which source countries might benefit from bioprospecting. Gordon pushed the whole collecting phase of the Natural Products Branch to help grow research capacity in the most biodiverse countries, where trained scientific personnel and research resources are always much too scarce. Dozens of international scientists came either to NCI, to the contracted collecting institutions, or to other leading research centers with NCI support for training to support their continued professional development. In country after country, I have met numerous scientists who all have been supported in their scientific growth, both financially and also with encouragement, by Gordon.

On November 10–11, 2006, a group of researchers convened in St. Louis at the Missouri Botanical Garden to honor Gordon Cragg and celebrate his recent retirement as Chief of the Natural Products Branch of NCI with two days of presentations. In one way or another, Gordon Cragg had touched and influenced everyone in attendance at the symposium. The talks from that event are presented in this volume, and collectively they present an overview of the present state of natural products research and a vision for its future.

Acknowledgments

This volume grew out of a symposium held November 10–11, 2006, at the Missouri Botanical Garden in St. Louis. Participants came from around the world to present their ideas and to honor Dr. Gordon Cragg of the National Cancer Institute. The symposium was organized by:

Flora N. Katz	Fogarty International Center, National Institutes of Health
David G. I. Kingston	Virginia Polytechnic Institute and State University
James S. Miller	New York Botanical Garden
David J. Newman	National Cancer Institute
Joshua P. Rosenthal	Fogarty International Center, National Institutes of Health

During the symposium, Dr. Cragg was presented with the 2006 William L. Brown Award for Excellence in Genetic Resource Conservation in recognition of his lifetime achievement in the field of natural products drug discovery. The William L. Brown Award is made possible through a generous endowment from The Sehgal Family Foundation, in cooperation with the Brown family.

Finally, the symposium would not have taken place without the liberal financial support of the following:

The Edward Mallinckrodt Foundation
Sequoia Sciences
Missouri Botanical Garden

The Natural World:
A Wealth of Chemical Diversity for Drug Discovery

Marine-derived Compounds: New Frontiers in Production

David J. Newman[1]

Perhaps the major stumbling block in the utilization of marine-based (sourced) compounds as drugs/leads to drugs has been the problem of "resupply." Although this was a genuine concern between the early 1970s and the late 1990s, nowadays it is feasible to "produce" marine-derived compounds in relatively large quantities and under cyclic guanosine monophosphate (cGMP) conditions, thanks to recent advances in genomics, microbial and invertebrate cultivation, and a quantum leap in synthetic chemistry techniques. Examples ranging from in-sea and land-based aquaculture, through fermentative production under saline conditions, to use of synthetic methods will be discussed. All of these are now proving to be of use in "utilizing the secrets of biodiversity."

Introduction

Although the reports of marine-derived compounds having significant activities in many pharmacologic areas have been well documented over the years, particularly in the extensive reviews initiated by the late D. John Faulkner and now continued by a New Zealand marine group (Blunt et al., 2006), there has been a perception, admittedly based on reality of ten plus years ago, that the development of marine-based compounds would require immense efforts from the aspect of recovery and processing of large amounts of biomass in order to obtain even enough material for initial in vivo screening, with very large amounts being necessary for further development processes where requirements might well be in the hundreds of grams to multi-kilogram levels of pure compound.

The perception was not helped by the fact that most of the agents discovered from marine sources are exquisitely potent, often with IC_{50} values in the nanomolar range, and are present as a result of the levels of activity, at very low levels in the invertebrate. Thus, published data such as 18 g of bryostatin 1 from over 13 metric tons of *Bugula neritina* or 300 mg of halichondrin B from 1 metric ton of a deep-water *Lissodendoryx* sp. did not help sell the concept of "drugs from the sea" to pharmaceutical houses.

Although true in years gone by, today, due to advances in biology and chemistry over the last five or so years, the situation is undergoing a "sea change," with possible methods/routes now being investigated as part and parcel of the initial discoveries.

In order to put the recent advances in perspective, below I will give some brief examples from the early days of marine-sourced agents in anticancer therapies (due to the majority of the funding being from the National Cancer Institute [NCI]), followed by exemplars from the intermediate (ten or so years old) and then the more modern approaches, ending with a discussion of the role(s) of microbes in the production and development of marine-sourced agents.

The Early Compounds

Didemnin B (Wild Collections and Syntheses)

The first compound to go into human clinical trials directly from a marine source was the cyclic depsipeptide, didemnin B (Figure 1) isolated by the Rinehart group from the encrusting tunicate *Tridemnum solidum* (Rinehart et al., 1981). This was one of a number of very similar compounds isolated from the same organism and included a very close analogue differing by only two hydrogens, dehydrodidemnin B (DDB) or as it is now known, apli-

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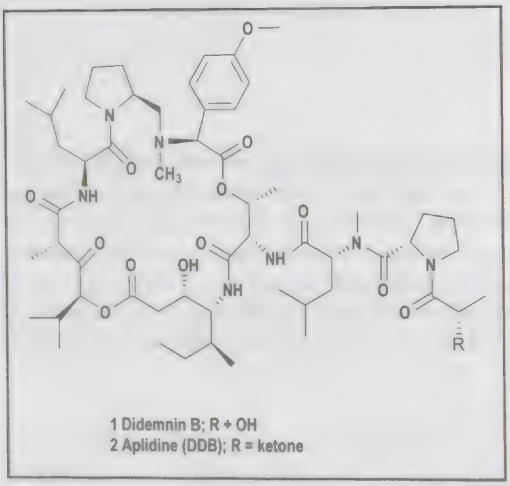

Figure 1. Didemnin B; Figure 2. Aplidine.

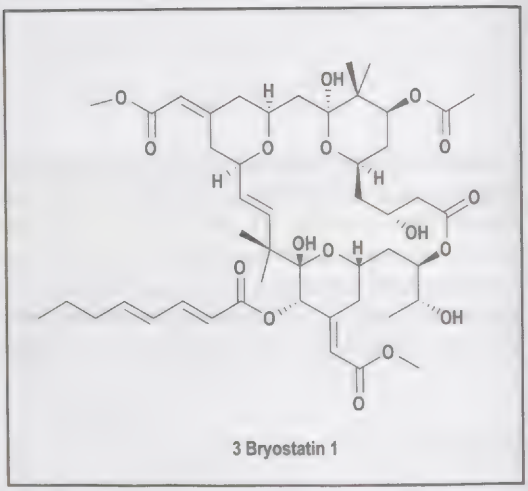

Figure 3. Bryostatin 1.

dine (Figure 2) (Henriquez, Faircloth, and Cuevas, 2005). This compound will be covered in greater detail later in the paper. Due to the paucity of the producing organism and the requirement for cyclic guanosine monophosphate (cGMP) materials for clinical trials, Rinehart's group in 1987 published a synthetic method with over seven further syntheses by the Schmidt, Shioiri, Joullie, and Jou groups with a discussion of these synthetic methods being given in the excellent review by Vera and Joullie (2002) that yielded a significant number of enantiomerically pure analogues together with the required didemnin B. Included in the 1997 report by Jou et al. (1997) was a route to DDB by liquid phase synthesis.

Didemnin B was placed into a significant number of clinical trials by the NCI but was finally taken off trials in the middle 1990s due to toxicities. What is significant, however, are the retrospective comments made by Vera and Joullie (2002) on the methods of delivery used in these early trials, a single bolus dose at close to the maximum tolerated dose (MTD). From later work with aplidine (Henriquez, Faircloth, and Cuevas, 2005), the use of different dosing schedules and a modicum of other supportive medications avoided the significant toxicities seen with didemnin B.

Bryostatins (Large-scale Collections, Aquaculture, and Potential Microbe)

The next compound to go into clinical trials was bryostatin 1 (Figure 3). Currently it has been in over 80, with one remaining trial still being listed on the NCI clinical trials web page. That this compound ever made it to human clinical trials can be ascribed to the determination of G. Robert (Bob) Pettit, who first reported on this class of compounds in 1970 (Pettit et al., 1970). Subsequent work, leading to the identification of around 19 variations on the same basic structure, has been reported in a large number of papers and reviews mainly as a result of the Pettit group and collaborators (Pettit, Herald, and Hogan, 2002), though recently a new bryostatin molecule, numbered as bryostatin 20, was reported from an Atlantic *Bugula neritina* by Lopanik, Gustafson, and Lindquist (2004) and is effectively the ring-closed version of bryostatin 10.

As a result of the initial preclinical and clinical (phase I) responses to bryostatin 1, it was necessary to produce gram quantities of the compound for initial trials. As a result of a tour de force by Program Resources Inc., the then NCI contractors at the NCI's Frederick Cancer Research Center at Fort Detrick, MD, 18 g of cGMP bryostatin 1 was produced by workup of 13,000 kg of wild-col-

lected *Bugula neritina* from the West Coast of the U.S. Details of the work were given in a variety of papers reviewed by Newman in 1996.

As a result of the problems involved in the original large-scale collection/isolation and the realization that if successful, a large amount of raw material would be necessary to produce the compound for clinical use, NCI funded a Small Business Innovation Research (SBIR) phase I (and a subsequent phase II) contract with the small California biotechnology company, CalBioMarine. The aims were to produce *Bugula neritina* by both in-sea and on-land aquaculture, in order to avoid the problems of seasonal variation in levels of this bryozoan. This was successfully completed, demonstrating that the organism could be aquacultured and that levels of bryostatin were high enough to obtain a supply of the compound, though significant optimization would have been necessary. The work involved was subsequently described by one of the CalBioMarine principals, Dominick Mendola (2003).

However, during this time span, neither synthetic chemists nor microbiologists were idle. A significant number of syntheses of parts of the bryostatin 1 molecule and total syntheses of various members of the complex were reported (reviewed by Norcross and Paterson, 1995; Mutter and Wills, 2000; Hale et al., 2002). In addition to regular syntheses, a number of "simplified" molecules were reported by Wender's group, taking as their first approach a potential model of where bryostatin 1 bound to protein kinase C, with a later paper covering the synthesis of modified B-ring "bryologs" (Wender, Horan, and Verma, 2006).

On the microbiological front, Haygood and her collaborators started to investigate the potential sources of bryostatins in both the adult animal and in the larvae. In a series of excellent investigations, they demonstrated that in the larvae of bryostatin-producing animals, there were as-yet-uncultured bacteria (given the name of *Candidatus Endobugula sertula* in the first report by Davidson and Haygood in 1999), from which the putative *bryA* cluster was reported in 2004 (Hildebrand et al., 2004). Further work by Lopanik et al. on Atlantic-sourced *Bugula neritina* has confirmed the presence of *bryA*-like fragments from their organisms as well, though in neither case does this prove the absolute production of bryostatins by the symbiont, but the evidence is highly suggestive (Lopanik, Targett, and Lindquist, 2006). Currently, the complete putative genomic sequence has been identified and sequenced but not yet expressed (Sudek et al., 2007). In addition, the location of the microbe throughout the complete life cycle of the bryozoan has now been reported (Sharp et al., 2007).

Dolastatins (Large-scale Collections, Syntheses, and Cyanobacteria)

The third marine natural product to go into clinical trials was the relatively simple pentapeptide, dolastatin 10 (Figure 4). This was isolated by Pettit's group from the Indian Ocean nudibranch, *Dolabella auricularia* (Pettit et al., 1987). Since the levels of this agent and its close relatives were very low, Pettit had to devise syntheses for the unusual aminoacids in the pentapeptide and then produce enough of the material for preclinical and clinical trials. In 1990, a potential mechanism for this agent was identified by Hamel's group, indicating that it interacted with tubulin at the *Vinca* alkaloid domain (Bai, Pettit, and Hamel, 1990). Details of the chemistry, pharmacology and clinical results with the dolastatins are given in reviews by Pettit (1997) and Flahive and Srirangam (2005), and currently none of the dolastatins themselves are still in clinical trial. However, the base structures have led to a multiplicity of analogues, one of which, tasidotin/synthadotin (Figure 5) is in phase I clinical trials for solid tumors, having earlier been in phase II trials for other tumor types.

What is of great interest from the aspect of this paper, however, is that in a series of papers from 1998 onwards, Paul and her colleagues demonstrated first the presence of a very simple modification of dolastatin 10 in the cyanobacterium *Symploca hydnoides* and then followed this with

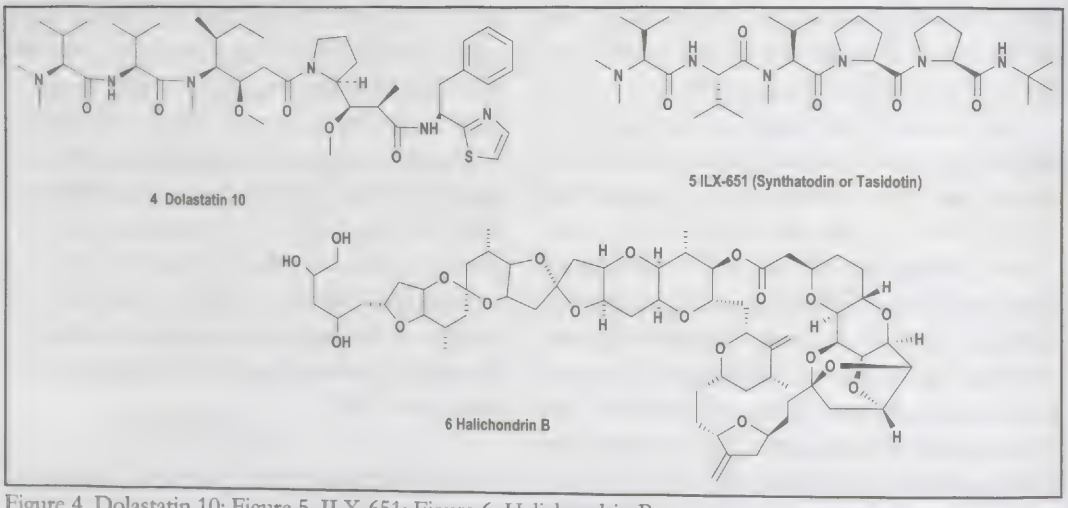

Figure 4. Dolastatin 10; Figure 5. ILX-651; Figure 6. Halichondrin B.

the isolation of dolastatin 10 itself from a closely related cyanobacterium, *Symploca* sp. VP642, an organism that *Dolabella auricularia* was seen to be grazing on (Luesch et al., 2001). Thus, there is now *prima facie* evidence for the microbial involvement in the production of dolastatin 10 and, by extension, a number of other closely related molecules, though further processing by the nudibranch cannot be ruled out at this stage.

Later Compounds

Halichondrin B and Analogues (Aquaculture and Chemical Syntheses)

Halichondrin B (Figure 6) was first reported from the Japanese sponge *Halichondria okadai* by Uemura's group in 1985 (Uemura et al., 1985) and 1986 (Hirata and Uemura, 1986) followed by reports of similar molecules from a variety of other sponge sources over the next few years. These initial reports were then followed by two essential papers, the first in 1991 showing that halichondrin B acted as a tubulin inhibitor potentially binding close to the *Vinca* site (Bai et al., 1991). The other was from Kishi's group at Harvard (funded by NCI) in 1992, giving a synthetic route to halichondrin B (Aicher et al., 1992).

As a result of the first of these reports and other work at NCI, in early 1992, the NCI's Decision Network Committee decided to recommend further preclinical development of halichondrin B. Now the real problems began because no simple source was known of the producing organism. With the help of the New Zealand marine chemistry group at the University of Canterbury and the National Institute of Water and Atmospheric Research (NIWA) in Wellington, together with very significant funding from NCI and the New Zealand government, a total of a little more than 1 metric ton of a deep-water *Lissodendoryx* sp. was harvested over the next four years and processed to yield 300 mg of 98%+ halichondrin B.

Over the same time frame, NCI financed a series of significant in-sea aquaculture experiments in New Zealand waters that demonstrated for the first time that a deep-water sponge could be aquacultured in shallow water and still produce a significant amount of the desired secondary metabolite. The initial reports were given in abstract format, but then a case study was published as part of a larger series of experiments by the New Zealand scientists directly involved (Munro et al., 1999).

Contemporaneously, a chemical group at the Eisai Research Institute in the U.S. was investigat-

ing the potential of the Kishi synthesis, having licensed the essential patents from Harvard. Fortuitously, their tumor biologist happened to see the work that Developmental Therapeutics Program (DTP) scientists reported on the in vitro activity of the pure halichondrin B at the 1998 American Association of Cancer Research (AACR) meeting, which led to a discussion of a collaborative project comparing the NCI's pure halichondrin B with two truncated, fully synthetic molecules that they had made, E7389 (Figure 7) and E7390 (Figure 8), which had the cyclic portion of halichondrin B with a very subtle modification of the ring, forming a ketone rather than the naturally occurring lactone, thus giving stability but maintaining activity. The full story of the synthetic approaches and the Eisai preclinical development of these two compounds was reported by the Eisai group in 2005 (Yu, Kishi, and Littlefield, 2005). Following in vitro and in vivo comparisons, E7389 was chosen as the better of the two synthetic analogues and was, in fact, significantly better than the natural product from a toxicity aspect.

The compound was approved by the NCI for clinical development in 2001, entered clinical trials soon thereafter, and is currently (12/09) in phase III clinical trials against refractory breast carcinoma. Its exact mechanism of action is still somewhat open as to the specific interaction with tubulin, but either a possible interaction with the monomer prior to attachment to the polymer or perhaps blockade of attachment of tubulin monomer have been suggested by Jordan (Jordan et al., 2005). Recently, molecular modeling techniques have been applied by an NCI group, giving information that suggests a similar binding site (Dabydeen et al., 2006).

Ecteinascidin 743 (Large-scale Collections, Aquaculture, Semisynthesis from Microbes)

This compound, ET743 (Figure 9) (trabectedin, Yondelis), has used all of the methods in the heading, and without the involvement of a microbial product, cyanosafracin B, it would not have reached its current stage of development with an application made to the European Medicines Agency (EMEA) in the EU (the equivalent of the U.S. FDA) for marketing approval in July 2006 as a treatment for sarcoma.

Though the antitumor activity of extracts of *Ecteinascidia turbinata* was first reported in 1969 (Sigel et al., 1969), the isolation and structure of ET743 were independently reported by the groups of Wright and Rinehart in 1990 (Rinehart Jr. et al., 1990; Wright et al., 1990). Inspection of the structures demonstrated that they contained the essential aspects of the known terrestrial anti-

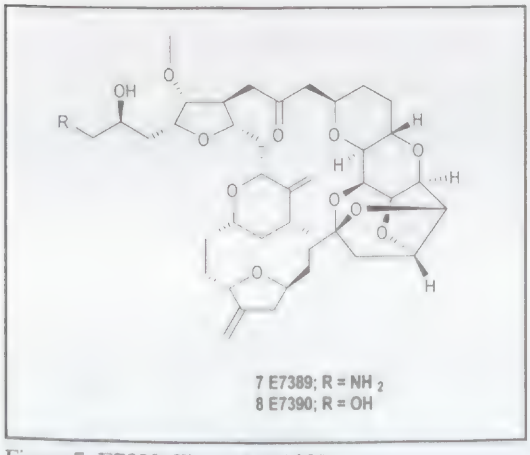

Figure 7. E7389; Figure 8. E7390.

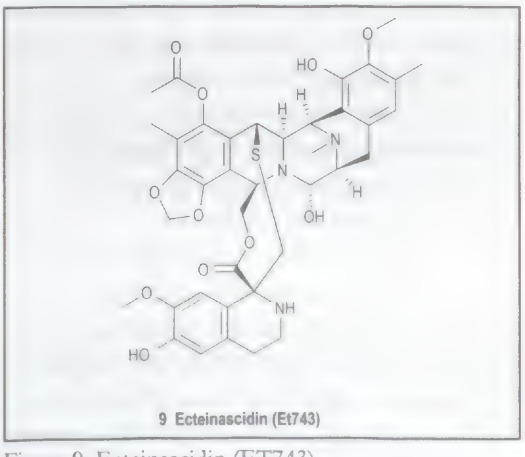

Figure 9. Ecteinascidin (ET743).

biotics of the saframycin class and that there were also other marine natural products with similar basic structures in the literature. A thorough and relatively recent review on the tetrahydroisoquinoline antitumor antibiotics should be consulted for further information on this general class (Scott and Williams, 2002).

ET743 was licensed to PharmaMar for development and over the next ten or so years, a whole variety of methods were utilized in order to obtain enough biomass to isolate the compound of interest. These included wild collections, in-sea and lake-based aquaculture, all of which provided enough material for preclinical and early clinical studies. However, in order to provide enough material to work with for advanced clinical and, if successful, commercial production, it became apparent that none of the current methods would suffice. Although Corey et al. had reported an enantioselective synthesis in 1996 (Corey, Gin, and Kania, 1996), it was not amenable to large-scale production; however, by using the marine bacterial product cyanosafracin B as the precursor, Cuevas et al. reported on a feasible route to cGMP production of ET743 (Cuevas et al., 2000). The full details of the further development through early 2005 have been reported by Henriquez et al. (2005). ET743 was approved by the EMEA in the EU in September 2007 for treatment of sarcoma and was launched in Sweden, Germany, and the United Kingdom by the end of 2007, thus becoming the first marine natural product to be an antitumor drug.

Aplidine/Dehydrodidemnin B (Wild Collections and Liquid-phase Peptide Synthesis)

As mentioned earlier when discussing didemnin B, a very close analogue, DDB or aplidine (Figure 2) was synthesized using solution-phase peptide synthetic methods (Jou et al., 1997). This compound had first been reported in a patent application by Rinehart, and then Urdiales et al. (1996) reported on the antiproliferative effect of this compound that had been isolated from the Mediterranean tunicate *Aplidium albicans*. The compound, now as the totally synthetic product, is currently in phase II clinical trials against a variety of cancers, and information through the middle of 2005 was given by Henriquez et al. (2005). Data on later clinical trials have been reported in abstract form at both the 2007 and 2008 AACR and American Society of Clinical Oncology meetings, with fuller details to come in due course.

Kahalalide F (Wild Collection and Solid-phase Peptide Synthesis, Algal Metabolite)

PharmaMar also has a third compound in clinical trials, Kahalalide F (Figure 10), which was isolated from a sea slug, *Elysia rufescens*, and, as in the case of the dolastatins, is an ingested product from another organism, this time an alga (*Bryopsis pennata*). Whether or not there is a smaller organism involved at the algal level is under investigation, but no reports have yet been made. However, it was obvious from the start of development that large-scale wild collections of the invertebrate would be impos-

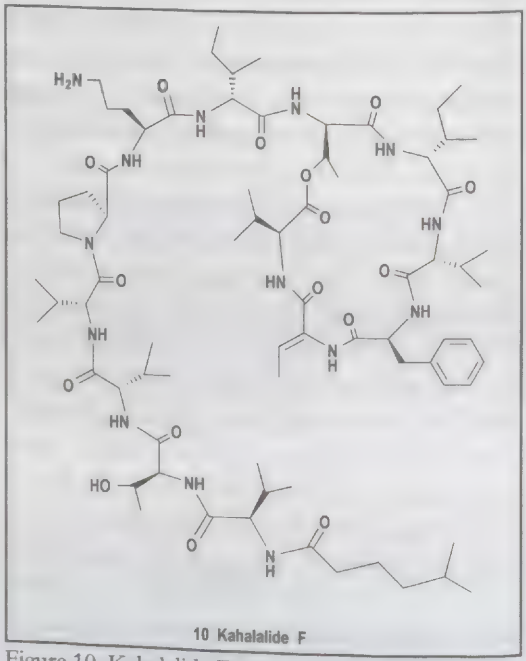

Figure 10. Kahalalide F.

sible and the levels in the alga were too small to be economically harvested, so the synthetic method of Jou et al. (1997) (a solid-phase technique) was utilized for supply. Currently this compound is in phase II clinical trials, and data up through early 2005 were given in the review by Henriquez et al. (2005). Recently, results from a phase I trial in patients with advanced solid tumors was reported (Pardo et al., 2008).

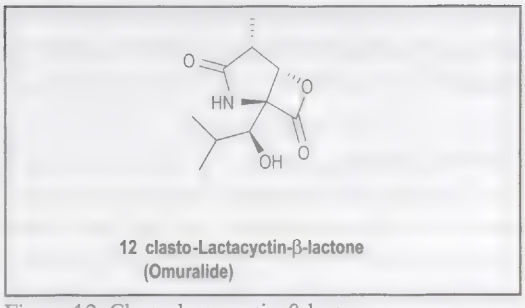

Figure 12. Clasto-lactacyctin-β-lactone.

Salinosporamide A (Marine Microbial Product and Fermentative Production)

This particular compound (Figure 11) is a trend-setter in the development of marine-derived molecules as it is from a new genus (*Salinispora*) of obligately marine bacteria found within the tropics and has both a unique structure and a mechanism that has only recently been proven to be a useful antitumor mechanism.

In 2003, Fehling et al. (2003) reported the structure and mechanism of action (proteasome inhibition) of salinosporamide A. The compound was relatively close in structure to the original terrestrial bacterial proteasome inhibitor omuralide (Figure 12) but differed in its substitution pattern from the structure activity relationships (SAR) established by Corey's group in their work with omuralide. The compound was licensed to Nereus Pharmaceuticals, who subsequently contracted with IRL in New Zealand to produce the compound by saline fermentation, establishing both an adequate production level and isolation and purification under cGMP conditions.

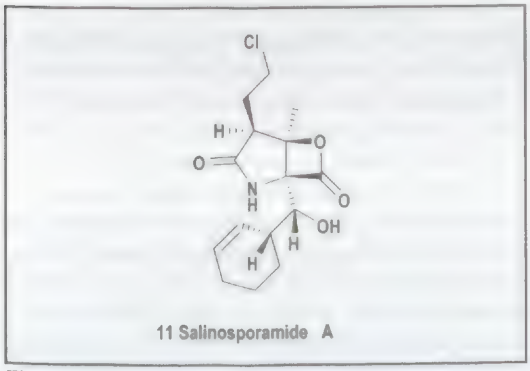

Figure 11. Salinosporamide A.

Contemporaneously, the chemistry groups at Nereus and Fenical's laboratories were synthesizing around the basic structure in order to establish SAR (Macherla et al., 2005) and isolating other analogues from the fermentations (Williams et al., 2005). The compound entered phase I clinical trials in the middle of 2006, just over three years from the original report of the isolation and activity, and a stereoselective formal synthesis of the molecule was reported by Caubert et al. (2007), with the first results from a phase I clinical trial presented in 2007 (Kurzrock et al., 2007).

In Conclusion

As can be seen from this relatively short review, the field of marine-derived antitumor agents has overcome its original production and perception problems, which could be best expressed as:

> *Very potent and interesting compounds but impossible to obtain by collection and/or synthesis.*

By utilizing all of the many techniques that have become available over the last ten or so years, from advances in aquaculture through realization that a large number of agents once thought to be invertebrate products are now considered to be, at a minimum, the products of interactions with microbes or, in some cases (e.g., the dolastatins and salinosporamides), definitively microbial in origin,

it is proving possible to rapidly advance the field in its supply and production aspects. Obviously the clinical trials cannot be rushed, but at least there are now adequate supplies available in an increasing number of cases.

To add to the compounds referred to above, there is a fairly recent short review that covers these and other marine-derived compounds that were in clinical trials, are currently in trials, or are in advanced preclinical studies from marine sources. This can be consulted for further information by the interested reader (Newman and Cragg, 2006).

What was not covered in any detail during the presentation upon which this article is based was the immense potential for genetic manipulation as both an isolation/purification tool, and also as a production tool for an increasing number of compounds from marine sources. That microbes are intimately involved, even though at times no microbes have yet been reported, can be seen by reading the reviews by Piel on the interrelationships of pederine from the blister beetle and the onnamides from a Japanese sponge (Piel, 2002, 2004; Piel et al., 2004), together with other possibilities related to use of genetically derived information in the production of interesting metabolites (Piel, 2006a, 2006b).

In closing, two relatively recent articles, the first a review in 2006 (Newman and Hill, 2006) and the second a paper in 2007 by Udwary et al. (2007), may serve to demonstrate how the microbial world is becoming increasingly important in any consideration of source/production of marine-derived compounds in multiple disease/non-disease areas.

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Amphibian Skin:
A Remarkable Source of Biologically Active Natural Products

John W. Daly[1†]

Biomedical research has greatly benefited from the influx of natural products with unique biological properties. Such natural products have provided antimicrobials, antifungals, analgetics, anti-inflammatories, antidiabetics, and anticancer agents. Natural products also have had an even greater impact as research probes to delineate the nature and function of enzymes, receptors, second messenger systems, ion channels, and transporters. Amphibian skin has provided a great variety of important peptides and alkaloids. Over 800 lipophilic alkaloids have been characterized from the skin of frogs and toads of four amphibian families, namely the dendrobatid poison frogs of the Neotropics, the mantellid poison frogs of Madagascar, one genera of bufonid toads from South America, and one genera of myobatrachid frogs from Australia. Most of these alkaloids are sequestered unchanged from dietary arthropods, in particular ants, mites, beetles, and millipedes. The batrachotoxins and the cardiotonic pumiliotoxins target sodium channels, while the histrionicotoxins, decahydroquinolines, pseudophrynamines, certain tricyclics, and various izidines target the nicotinic receptor/channels as noncompetitive blockers. The potent analgetic epibatidine targets nicotinic receptors as a high-affinity agonist. Such alkaloids have had a major impact on biomedical research, and there remain many further alkaloids of amphibian skin requiring further structural and pharmacological characterization.

The plant, microbial, and marine worlds have provided a rich array of biologically active natural products that have had profound effects on biomedical research. The discovery of such natural products has relied on searches for unique chemical compounds or on searches for compounds with certain biological properties. Amphibian skin has provided antimicrobial peptides, a variety of biogenic amine congeners, toxic bufadienolides targeting Na/K ATPase, tetrodotoxins targeting sodium channels, and a wide structural variety of lipophilic alkaloids (Daly et al., 1987). Virtually all the lipophilic alkaloids have come from dendrobatid poison frogs of the Neotropics, mantellid poison frogs of Madagascar, one lineage of bufonid toads of South America, and one lineage of myobatrachid frogs of Australia (Daly et al., 2005). Several of these alkaloids have become major pharmacodynamic probes (Daly, 2003) (Table 1).

Batrachotoxin was isolated from skin extracts of Colombian poison-dart frogs of the dendrobatid genus *Phyllobates* (Figure 1) and became an important tool for the specific activation of sodium channels (Albuquerque et al., 1971). In the form of a radioactive ligand, it has also been used to probe interactions of local anesthetics, anticonvulsants, anti-arrhythmics, and other drugs with such channels (McNeal et al., 1985). Batrachotoxins have now been found in the feathers and/or skin of two species of birds from Papua New Guinea and in a putative dietary source, a small red and black melyrid beetle (Dumbacher et al., 1992, 2000, 2004). The discovery of the batrachotoxins in frogs was due to the use of the skin secretions by Colombian

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[1]The considerable contributions of biologist Charles W. Myers, chemist Takashi Tokuyama, x-ray crystallographer Isabella L. Karle, pharmacologists Edson X. Albuquerque and Fabian Gusovsky, and especially my two current chemist colleagues H. Martin Garraffo and Thomas F. Spande are gratefully acknowledged. Many others have been major contributors over the past four decades, supported by the intramural program of the National Institute of Diabetes and Digestive and Kidney Diseases. These include Michael Edwards, Poonam Jain, Tetsuo Kaneko, Barbara Badio, Ralph Saporito, and N. Rabe Andrianamaharavo.

	Peptides	Biogenic Amines	Bufadienolides	Tetrodotoxins	Lipophilic Alkaloids: Samandarines	Lipophilic Alkaloids: "Dendrobatid"
Taxa where these substances occur	Hylidae Ranidae Discoglossidae Myobatrachidae	Bufonidae Leptodactylidae Hylidae Myobatrachidae	Bufonidae: *Bufo* *Atelopus* *Ansonia* *Pedostipes*	Salamandrinidae Bufonidae Brachycephalidae Rhacophoridae	Salamandrinidae: *Salamandra*	Dendrobatidae Mantellidae Bufonidae Myobatrachidae

Table 1. Biologically active substances in amphibian skin.

Indians to poison blow darts, while the discovery of batrachotoxins in birds and beetles were in both cases based on local knowledge of the toxicity of such species.

Pumiliotoxins A and B were first isolated from skin extracts of a small dendrobatid poison frog from Isla Bastimentos, Panama (Daly and Myers, 1967) (Figure 2). The frog was known by herpetologists to be toxic to other frogs. The structures were finally elucidated in 1980 (Daly et al., 1980), and the pumiliotoxins were proposed to be a new class of cardiotonic agents (Daly et al., 1985, 1988), whose activity was due to positive modulatory effects on sodium channels (Gusovsky et al., 1988, 1992). Recently, it was discovered that certain dendrobatid poison frogs are capable of stereoselectively converting a dietary pumiliotoxin to a more toxic allopumiliotoxin (Daly et al., 2003). Pumiliotoxins probably are sequestered from dietary mites (Takada et al., Saporito et al., unpublished results) but also have been detected in formicine ants (Saporito et al., 2004).

Further research on alkaloids from amphibian skin has led to the discovery of over 800 alkaloids. Structural classes of amphibian alkaloids include the samandarines, batrachotoxins, histrionicotoxins, pumiliotoxins, allopumiliotoxins, homopumiliotoxins, decahydroquinolines, pyrrolizidines, various indolizidines, quinolizidines, epibatidines, and tricyclics such as precoccinelline, the pseudophrynamines, and spiropyrrolizidines (Figure 3). The samandarines were isolat-

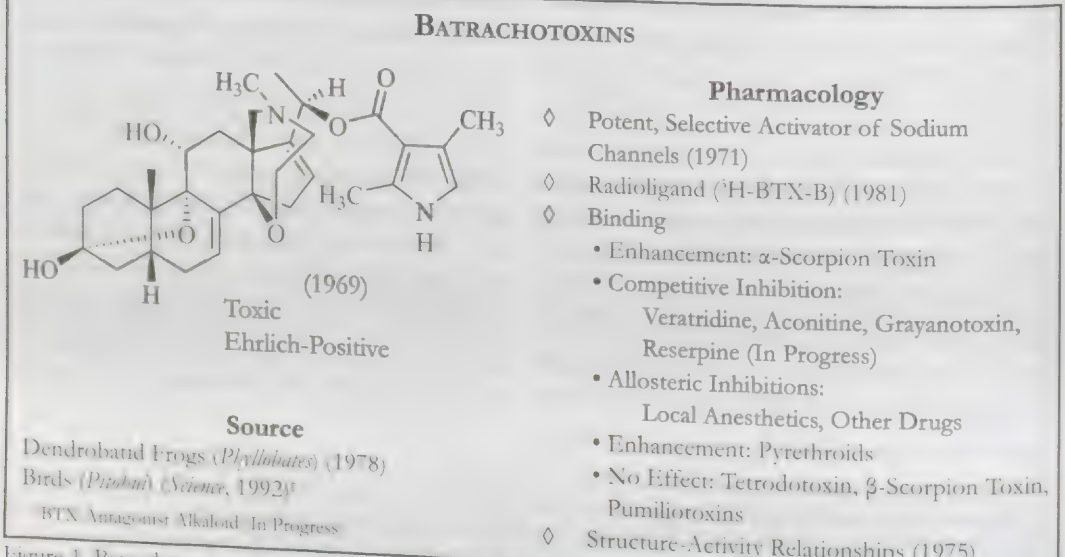
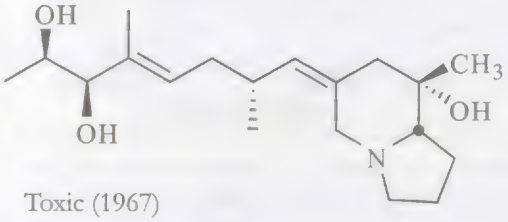


Figure 1. Batrachotoxins. Active alkaloids from skin of Colombian poison-dart frogs and Papua New Guinea birds. A putative dietary source is a melyrid beetle. Dates in parentheses that have no mention of a publication indicate the year John Daly made an initial discovery in the corresponding area of research.

PUMILIOTOXIN B



Toxic (1967)
Distinctive Mass Spectrum
Cardiotonic/Myotonic (1978)

Source

Frog and Toad Skin
(Dendrobatidae, Myobatrachidae, Mantellinae, and Bufonidae)

Pharmacology

- ◊ Positive Modulation of Sodium Channels (1988)
  - Potentiation: α- and β-Scorpion Toxins, Brevetoxins
  - Inhibition: Pyrethroids
  - No Effect on ^{3}H-BTX-B Binding but Antagonizes Inhibition by Aconitine (*Mol. Pharmacol.* 1992)
  - Enhances Phosphoinositide Breakdown (1991)
  - Proposal of an Alkaloid-Binding Domain (*Mol. Pharmacol.* 1992)
- ◊ Structure-Activity Relationships (1988, 1990)
- ◊ Enhances Release of Intracellular Calcium (1981 and In Progress)

Figure 2. Pumiliotoxins. Cardiotonic alkaloids from skin of many poison frogs and toads from the Neotropics, subtropical South America, Madagascar, and Australia. The putative dietary source is probably mites, but pumiliotoxins have also been detected in formicine ants. Dates in parentheses that have no mention of a publication indicate the year John Daly made an initial discovery in the corresponding area of research.

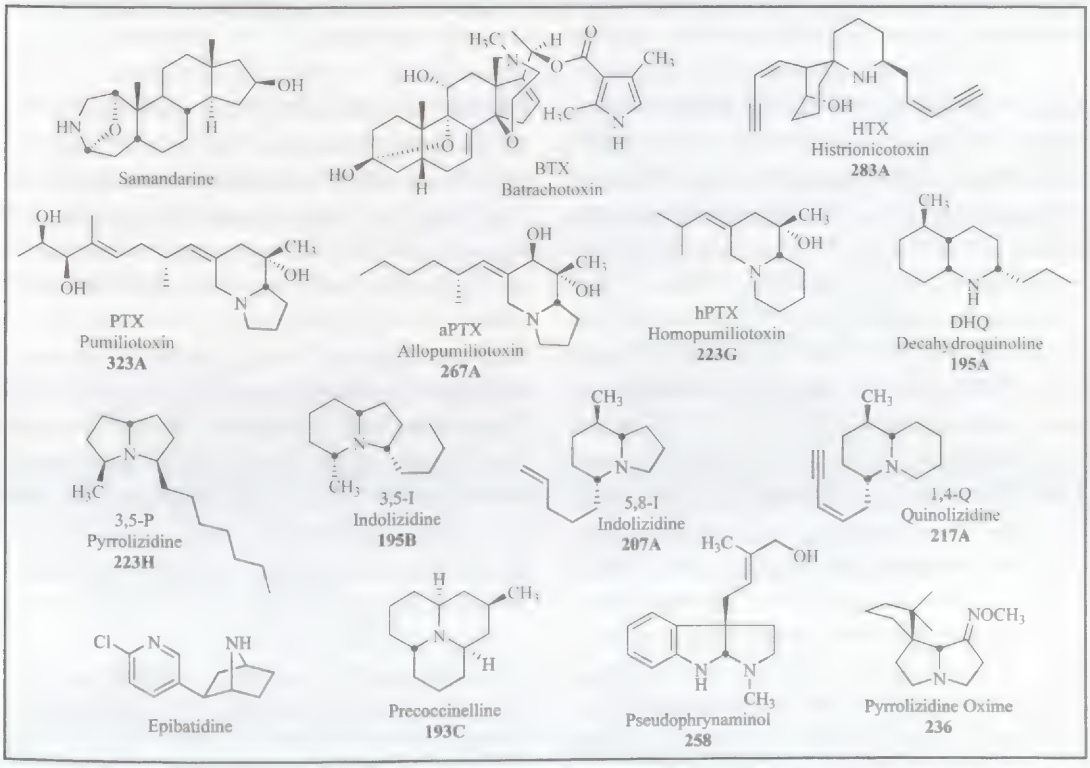


Figure 3. Representative alkaloids of some of the over 20 structural classes of alkaloids found in extracts of amphibian skin.

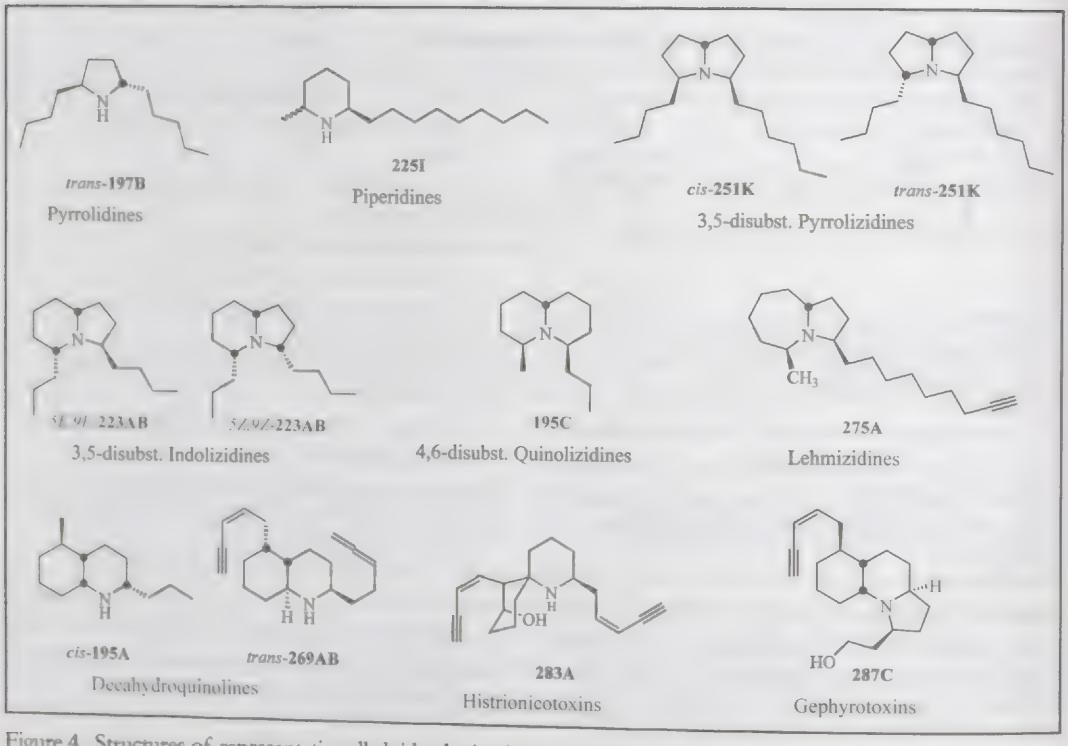


Figure 4. Structures of representative alkaloids obtained by amphibians, it has been proposed, from dietary myrmicine ants.

ed from the European Fire Salamander (Schöpf, 1961), the batrachotoxins only from dendrobatid poison-dart frogs of the genus *Phyllobates* (Tokuyama and Daly, 1983, and refs. therein), the histrionicotoxins only from the dendrobatid poison frogs of Central and South America (Daly, 2003), epibatidine only from certain dendrobatid poison frogs of the genus *Epipedobates* (Spande et al., 1992), and the pseudophrynamines only from myobatrachid frogs of the Australian genus *Pseudophryne* (Spande et al., 1988; Daly et al., 1990). The pumiliotoxins and allopumiliotoxins are found throughout all of the lineages of dendrobatid, mantellid, bufonid, and myobatrachid frogs that have lipophilic skin alkaloids (Daly, 2003). The decahydroquinolines, izidines, tricyclics, and spiropyrrolizidines are found in dendrobatids (Daly et al., 1987; Daly, 2003) and also in mantellid poison frogs (Garraffo et al., 1993b) and bufonid toads of the genus *Melanophryniscus* (Garraffo et al., 1993a). Remarkably, they are not found in myobatrachid frogs of the Australian genus *Pseudophryne*, whose skins contain only pumiliotoxins and/or allopumiliotoxins of dietary origin and the indolic pseudophrynamines that are produced by the *Pseudophryne* frogs (Smith et al., 2002). The histrionicotoxins, decahydroquinolines, and the izidines with unbranched carbon skeletons (Figure 4) are all suspected to come from dietary myrmicine ants (Jones et al., 1999). Coccinelline-like alkaloids are thought to come from beetles (Daly, 2003), while spiropyrrolizidine oximes come from millipedes (Saporito et al., 2003; Clark et al., 2005) (Figure 5). The pumiliotoxins and izidines with branched carbon skeletons are now thought to come from oribatid mites (Takada et al., 2005; Saporito et al., unpublished results). However, pumiliotoxins have also been found in formicine ants (Saporito et al., 2004).

The histrionicotoxins were the third structural class of dendrobatid alkaloids to be

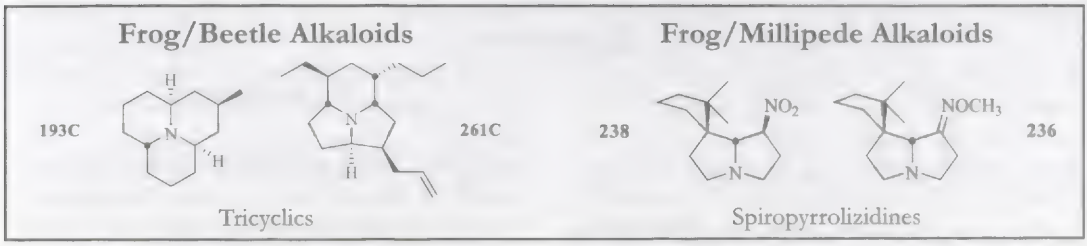


Figure 5. It has been proposed that tricyclic coccinelline-like alkaloids are obtained by amphibians from dietary beetles and spiropyrrolizidine alkaloids from a siphonotid millipede.

reported (Daly et al., 1971) (Figure 6). They proved to be "high-affinity" noncompetitive blockers of nicotinic channels (Spivak et al., 1982, and refs. therein) (Figure 7). The decahydroquinolines, izidines, pseudophrynamines, and spiropyrrolizidine oximes are also noncompetitive blockers of nicotinic receptors/channels (Daly, 2005).

Our knowledge of acetylcholine receptors has benefited greatly from research with natural products (Daly, 2005). The division of acetylcholine receptors into the nicotinic receptors, which are present as ion channels, and the muscarinic receptors, which are linked through G-proteins to second messenger systems, is due to the tobacco alkaloid nicotine and the mushroom alkaloid muscarine. Other alkaloids with nicotinic agonist activity include cytisine, anatoxin, ferruginine, anabaseine, and epibatidine (Daly, 2005). Antagonists include erysodine, methyllyaconitine, tubocurarine, and ibogaine. The alkaloid galanthamine is a positive modulator of nicotinic receptors.

Epibatidine was discovered in skin extracts of an Ecuadoran poison frog of the genus *Epipedobates* (Spande et al., 1992). The discovery was based on the observation that the alkaloid fraction from this frog caused a marked Straub tail, indicative of the presence of opioid- or nicotine-like activity. The extract contained a variety of pumiliotoxins and izidines, none of which were known to cause such activity. Epibatidine

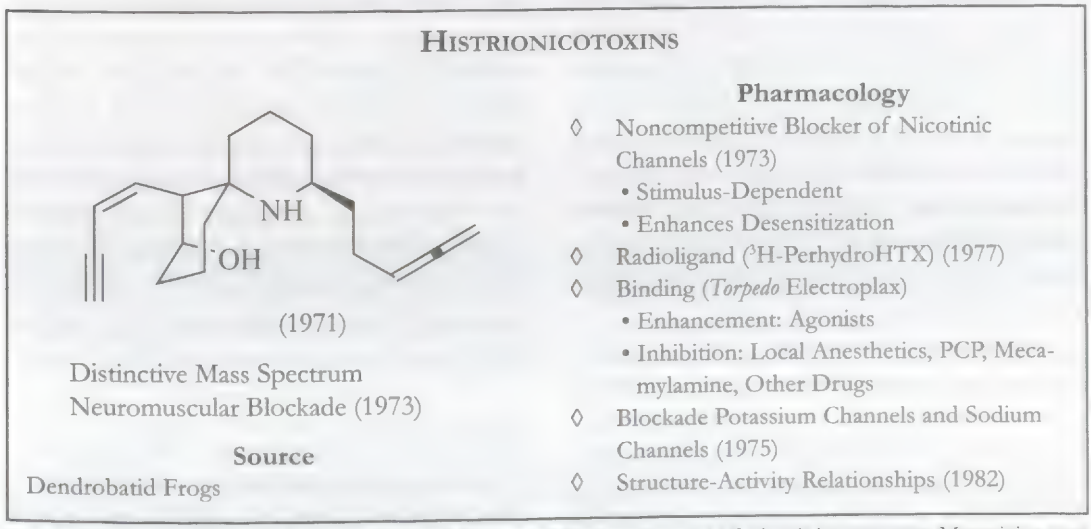
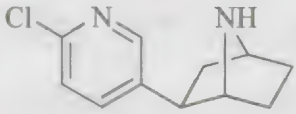


Figure 6. Histrionicotoxins. Noncompetitive blockers of the ion channel of nicotinic receptors. Myrmicine ants are the suspected dietary source. Dates in parentheses that have no mention of a publication indicate the year John Daly made an initial discovery in the corresponding area of research.

EPIBATIDINE



(*J. Am. Chem. Soc.*, 1992)

Straub-tail (1978)
Antinociceptive (1980)
Toxic

Source

Dendrobatid Frogs (*Epipedobates*)

Pharmacology

◊ Potent Nicotinic Agonist (*Mol. Pharmacol.* 1994)
- Selective $\alpha_3\beta_{4(5)}$, $\alpha_4\beta_2$ Subtypes
- Lack Enantioselectivity

◊ Radioligand (^{3}H-Epibatidine)

◊ Structure-Activity Relationships (*Mol. Pharmacol.* 1994; *Drug Devel. Res.* 1995)

Figure 7. Epibatidine. Potent analgetic due to nicotinic agonist activity. Isolated from skin extracts of an Ecuadoran dendrobatid frog of the genus *Epipedobates*. A dietary source is unknown. Dates in parentheses that have no mention of a publication indicate the year John Daly made an initial discovery in the corresponding area of research.

(Figure 7) proved to be a potent analgetic and nicotinic agonist (Badio et al., 1994).

Synthetic efforts in many laboratories have led to many nicotinic agonists, some of which have reduced the toxicity, while retaining the potent analgetic activity of epibatidine (Daly, 2005, and refs. therein). Some have entered clinical trials, like ABT-594 (Bannon et al., 1998).

An alkaloid fraction from another population of the same frog was fractionated by high-performance liquid chromatography and the nicotinic activity of the fractions monitored by a calcium fluorescent assay in cells expressing nicotinic receptors (Fitch et al., 2003). Epibatidine was the major nicotinic agonist. *N*-Methylepibatidine, another chloropyridyl-containing alkaloid named phantasmidine, and the novel quinolizidine epiquinamide were also isolated (Daly et al., 2005).

Further research on alkaloids from amphibian skin and on arthropods as potential sources of such alkaloids is in process. Characterization of many of the over 800 alkaloids is incomplete. Most have not been isolated and are present in extracts containing mixtures of 20 to 80 alkaloids. Thus, characterization often has been based only on gas chromatographic spectral analyses (Electron Ionization Mass Spectrometry [EI-MS] alone or in tandem [EI-MS/MS]; Chemical Ionization MS [CI-MS] alone or in tandem after collision-activated ionization [CI-MS/MS]; CI-MS after deuterium exchange with deuteroammonia; high-resolution EI-MS; vapor phase Fourier transform infrared spectroscopy [GC-FTIR]) on the alkaloid fraction either before or in some cases after on-line *N*-methylation or *N*-acetylation or after perhydrogenation. After isolation nuclear magnetic resonance spectroscopic analyses have defined many structures. The challenge now is to isolate alkaloids present as trace components (1–100 μg) in alkaloid fractions composed of complex mixtures with major, structurally defined alkaloids often present in much larger amounts. Many extracts from dendrobatid, mantellid, and bufonid anurans are currently being subjected to analyses of alkaloids both in mixtures and after isolation.

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Bioprospecting Uncultured Microbial Biodiversity

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Bioprospecting for bioactive small molecules from microbial sources has been a hugely successful endeavor. Thousands of compounds have been identified, mainly from *Actinomyces*. The rate of discovery of novel compounds has, however, slowed precipitously in recent years. This slowdown is especially pronounced for novel antibiotics, which are critically needed in clinical settings due to the emergence of resistant pathogens. This article discusses the reasons for a reduced rate of discovery and describes methods that may help to enhance the productivity of future, cultivation-based microbial bioprospecting endeavors. Diverse or unique actinomycete communities can be identified using the culture-independent technologies currently used in microbial ecology. High-throughput screening and molecular comparison of isolates can reduce redundant analysis of isolates. More than 99% of bacteria in the environment cannot be grown in standard isolation media, excluding the vast majority of bacterial diversity from traditional bioprospecting. Recent advances in the relatively nascent field of metagenomics promise access to the genetic material of these uncultured microbes. Direct cloning of environmental DNA can be used to obtain their secondary metabolite genes. The metagenomic literature is reviewed for approaches, methodologies, and advances in identifying novel antimicrobial compounds. Approaches for identifying unexpressed biosynthetic pathways are discussed. We conclude that the tools of microbial ecology may reinvigorate natural products discovery by allowing more targeted surveys, increased productivity, and a reduced rate of rediscovery.

The Demise of Antibiotics

Small molecules directly or indirectly derived from natural products have historically been the most important source of drugs used in chemotherapy (Koehn and Carter, 2005). The heyday of natural products discovery was during the 1970s and 1980s. Since then, there has been a relative decline in original natural product patents as a function of research expenditure (Koehn and Carter, 2005). This decline in research effort can in part be attributed to the development of high-throughput technologies allowing the application and screening of large combinatorial chemistry libraries. Such libraries are highly adaptable to high-throughput screening, contain a very large diversity of chemical structures, and avoid potentially difficult legal negations with the originators of biological material. Combinatorial chemistry also offers the added advantage of a clear path to synthesis and thus an unlimited supply of active compound. The other factor leading to the decline has been a reduced emphasis on drugs used in the treatment of infectious disease (Figure 1). Natural products are especially relevant in this area, because many active molecules, especially those from microbial sources, have coevolved as antagonistic agents. This decreased interest may seem surprising considering that global annual sales of antibiotics exceed $30 billion (Bentley, 1999; Demain, 2000;

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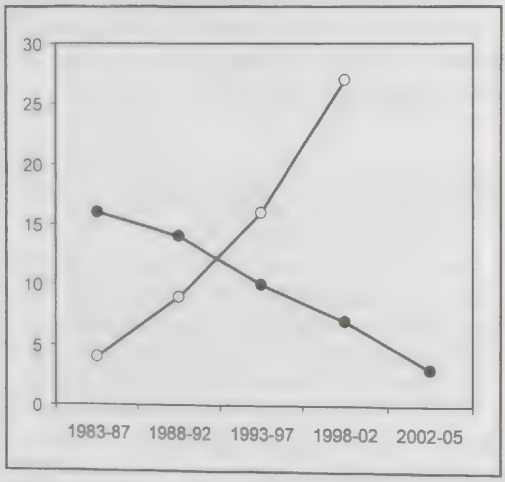

Figure 1. Trend in total research and development (R&D) expenditure and antimicrobial drug approval rate. (○) Total, yearly, inflation-adjusted R&D expenditure in billion dollars (2000 equivalent) of U.S. pharmaceutical companies. Numbers were adapted from DiMasi, Hansen, and Grabowski (2003). (●) New antibacterial agents approved by the Food and Drug Administration (FDA) in the U.S. between 1983 and 2005. Data were adapted from von Nussbaum et al. (2006).

DiMasi, Hansen, and Grabowski, 2003). However, not one of the top 20 selling drugs in the U.S. is an antibiotic. Drugs that affect high cholesterol, heartburn, asthma, depression, allergies, or erectile dysfunction each produce billions of dollars in annual sales and revenue. Total annual U.S. antibiotic sales in 1995 were approximately $8 billion (Demain, 1999), roughly equivalent to Pfizer's 2005 sales of Lipitor alone ($8.4 billion) (Herper, 2006). If one considers that bringing a drug to market costs as much as $800 million (DiMasi, Hansen, and Grabowski, 2003), it is apparent that greater profitability can be achieved by developing drugs for chronic disorders, rather than infectious disease.

By itself, a slowdown in the development of novel antibiotics may not seem significant. Since the 1940s, a large number of antimicrobial agents, attacking virtually every component of microbial cellular activity, have been discovered. More than 6,000 antibiotics have now been described, and new chemicals are still being added to the list at an appreciable rate (Demain, 2000). About 160 of these compounds are currently being sold in the market (Demain, 2000), finding applications in human health, livestock, and aquaculture. The societal benefits have been tremendous. More importantly, there has been a dramatic reduction in mortality caused by bacterial infections. In 1900, approximately 30% of all mortality in the United States was directly caused by infectious disease (Lederberg, 1997). This number dropped precipitously over the first 60 years of the 20th century. One hundred years later, only 3.1% (75,000) of deaths in the United States were caused by microbial agents (Mokdad et al., 2004), which is less than those caused by the consumption of alcohol (3.5%). The leading five causes of death in 2002 were heart disease, cancer, stroke, respiratory disease, and accidents; together these account for 67% of all mortality and 25 times the number of deaths caused by influenza and pneunomia, the leading causes of microbial mortality (Anderson and Smith, 2005). While other factors such as improved hygiene, healthcare, education, and public services have played major roles in improving public health, the most important factor has been the widespread use of antibiotics. Few people today worry that bacterial infections may become life threatening. Public and corporate interest in developing new antimicrobial drugs has concomitantly waned. Compounded with reduced relative research efforts resulting from the practical and economic circumstances pointed out above, an "innovation gap" of several decades has developed, and few new antibiotics are now being introduced into the market (von Nussbaum et al., 2006).

The significance of this "innovation gap" can be appreciated when recent trends in the incidence of antibiotic-resistant bacterial strains isolated in clinical settings are considered. Infectious disease is, in fact, on the rise again. An ever greater number of clinical isolates are resistant to at least one, sometimes several, and in a few, still

rare cases, all antibiotics available for chemotherapy. For example, by 2003 89% of coagulase-negative staphylococci isolates associated with nosocomial infections in ICU patients were methicillin resistant (Anonymous, 2004). The same study reports that nearly 60% of *Staphylococcus aureus* were methicillin, oxacillin, or nafcillin resistant, and that the incidence of *Klebsiella pneumoniae* not responsive to third-generation cephalosporins had risen by nearly 50% between 2002 and 2003 (Anonymous, 2004). The causes of increased resistance are varied and include over-prescription, misuse, and incompetence of hospital staff. The primary cause, however, is the use of antibiotics itself. The emergence and spread of antibiotic resistance is in essence a fascinating, global demonstration of Darwinian principles of evolution. When new antibiotics are deployed, they create enormous selective pressure on bacterial populations to evolve, acquire resistance, and thus adapt to their new environments. Once resistance has been acquired, it is rarely lost. Our only option for continued effective treatment becomes development of new antibiotics. This engages us in a perpetual arms race to outpace the emergence of bacterial resistance with the development of new drugs. We are currently losing this race. Antibiotics that were once considered last resort treatments are now often the primary drug of choice leaving no last-resort if chemotherapy fails (von Nussbaum et al., 2006). New and effective antibiotic drugs are thus urgently needed.

Bioprospecting Microbes in the Environment

Fewer than 1% of all known organic molecules are of natural products origin (von Nussbaum et al., 2006), yet more than two thirds of all drugs currently sold are directly derived or were inspired by natural products (Newman, Cragg, and Snader, 2003). The statistics are even more lopsided for antibiotics. Seventy-nine percent of all antibacterial new chemical entities (NCE) submitted in the two decades after 1984 have been derived from natural product lead structures (von Nussbaum et al., 2006). Several theories have been put forth to explain the advantages of natural products–derived NCEs over combinatorial chemistry as potential drug leads. In the case of antibiotics, however, the advantages are obvious. The molecules evolved for the very purpose that makes them attractive to us: the ability to inhibit the growth of bacteria.

The most prolific source of antibiotics has been microbes themselves, and two thirds of all known antibiotics originate from a single class of soil bacteria, the actinomycetes (Demain, 2000). Two factors are responsible for this bias toward actinomycetes. First, actinomycetes appear to be especially prolific producers of secondary metabolites. *Streptomyces coelicolor* A3(2), for example, contains at least 20 different gene clusters for secondary metabolite production, including type I and II polyketide synthases (PKS), chalcone synthases, non-ribosomal peptide synthetases (NRPS), terpene cyclases, and others (Bentley et al., 2002). The second reason is the high culturability of actinomycetes. A large proportion of actinomycete species in the environment appears to be amenable to cultivation, while several lines of evidence indicate that cultivable bacteria are not representative of microbial diversity in the environment as a whole. This was first recognized when culture collections were compared to ribosomal RNA gene sequences cloned from the environment (Olsen et al., 1986; Pace et al., 1986; Pace, 1997; Hugenholtz, Goebel, and Pace, 1998) and has since been confirmed by many other studies. It is now known that microbial communities in the environment are mostly composed of members not found in culture collections. Thousands of different and unknown bacterial ribosomal RNA gene sequences have been obtained from the environment, revealing dozens of previously unknown bacterial divisions. This is akin to a zoologist discovering that most animal phyla had not been known to science. Actinomycetes as a group grow well under laboratory conditions. In a survey of 3,000 16S ribosomal RNA gene se-

quences obtained from studies of environmental samples, it was found that approximately 80% of actinomycete-like sequences were similar to those of cultivated species (Hugenholtz, Goebel, and Pace, 1998). Actinomycetes thus have the highest proportion of cultivated members of any bacterial group, which has important implications for bioprospecting work. In fact, the cultivation of actinomycete species has been so successful that it is now difficult to isolate previously unknown species. The chance of rediscovering an already known secondary metabolite from an actinomycete isolate is now 99% (Zaehner and Fiedler, 1995; Handelsman et al., 1998). Microbial bioprospecting thus currently faces two important challenges: (a) how to efficiently isolate the remaining 20% of actinomycete species, and (b) how to access the secondary metabolism of the vast and uncultivated majority of microbes found in the environment. Recent advances in molecular biology and microbial ecology have provided exciting new opportunities to address these two questions, and the remainder of this article will focus on such methodologies.

Improving Cultivation-based Microbial Bioprospecting

Traditionally, bioprospecting for microbial secondary metabolites has involved the isolation of individual bacterial strains from single colonies on soil dilution plates, which favor the growth of actinomycetes (e.g., starch-based medium). Actinomycete strains are then grown in large-batch cultures and extracted. Extracts are tested for activity, and positive extracts are fractionated and analyzed by means of high-performance liquid chromatography (HPLC) and/or gas chromatography-mass spectrometry (GC-MS) analysis to determine the identity of active components. This approach has been very successful, and over 17,000 biologically active molecules have been described from prokaryotic isolates (Roberts and Higton, 1988; Glasby, 1992; Borders, 1999). As already pointed out, however, the rediscovery rate of already known structures is now in the range of 99% (Zaehner and Fiedler, 1995; Handelsman et al., 1998). Combined with the reality that very few initial "hits" advance to the lead compound stage and even fewer will make it to clinical testing, where there is still considerable risk of failure, it is evident that this approach to bioprospecting is likely to yield a very low return on any investment of time, effort, and resources. Unlocking the secondary metabolism of cultivable, but perhaps less abundant and more difficult to detect, actinomycetes will, therefore, require dramatic increases in throughput and efficiency of screening methodologies. Such increases should not be associated with a concomitant increase in cost. In other words, a large increase in productivity is needed to make bioprospecting of actinomycetes a viable option in the future. An important focus of the Central Asia International Cooperative Biodiversity Group (ICBG) Prokaryotic Microorganisms Associate Program (PMAP) group (http://aesop.rutgers.edu/~icbg/) has been to develop methodologies that increase efficiency and throughput, lower cost, and provide a more targeted approach to sampling. Particularly useful in this regard are modern tools of molecular biology and microbial ecology. Figure 2 illustrates how the productivity of microbial bioprospecting might be improved: (A) the tools of microbial ecology make it possible to prescreen soil samples and identify those containing particularly high diversity or unique community members; (B) direct screening for the identification of colonies that produce antibiotics inhibitory to engineered challenge strains; (C) molecular characterization of active isolates allowing for the identification of those strains that contain novel and unknown secondary metabolite pathways.

Screening for Unique Actinomycete Communities

A basic tenet of microbial ecology for the past century has been that all bacterial species can es-

sentially be found everywhere, that dispersal is high, and that natural selection creates the communities we observe in the environment (Beijerinck, 1913; Dykhuizen, 1998; Cho and Tiedje, 2000). This paradigm, known as the "everything is everywhere" hypothesis, has in recent years come under scrutiny. Data from experiments using sensitive molecular techniques have shown that microbial species are not homogenously distributed on a global scale and that high levels of strain endemism may exist in the environment. For example, cyanobacterial communities in thermal hot springs have been studied to determine their level of biodiversity and endemism (Castenholz, 1996; Papke et al., 2003). It was discovered that some strains found in North American springs were absent from springs in Alaska and Iceland (Castenholz, 1996). In a molecular survey of North American, Japanese, New Zealand, and Italian springs, it was found that phylogenetic analysis was consistent with geographic isolation on the global and local scales (Papke et al., 2003). The physical and chemical parameters of the springs could not account for the dis-

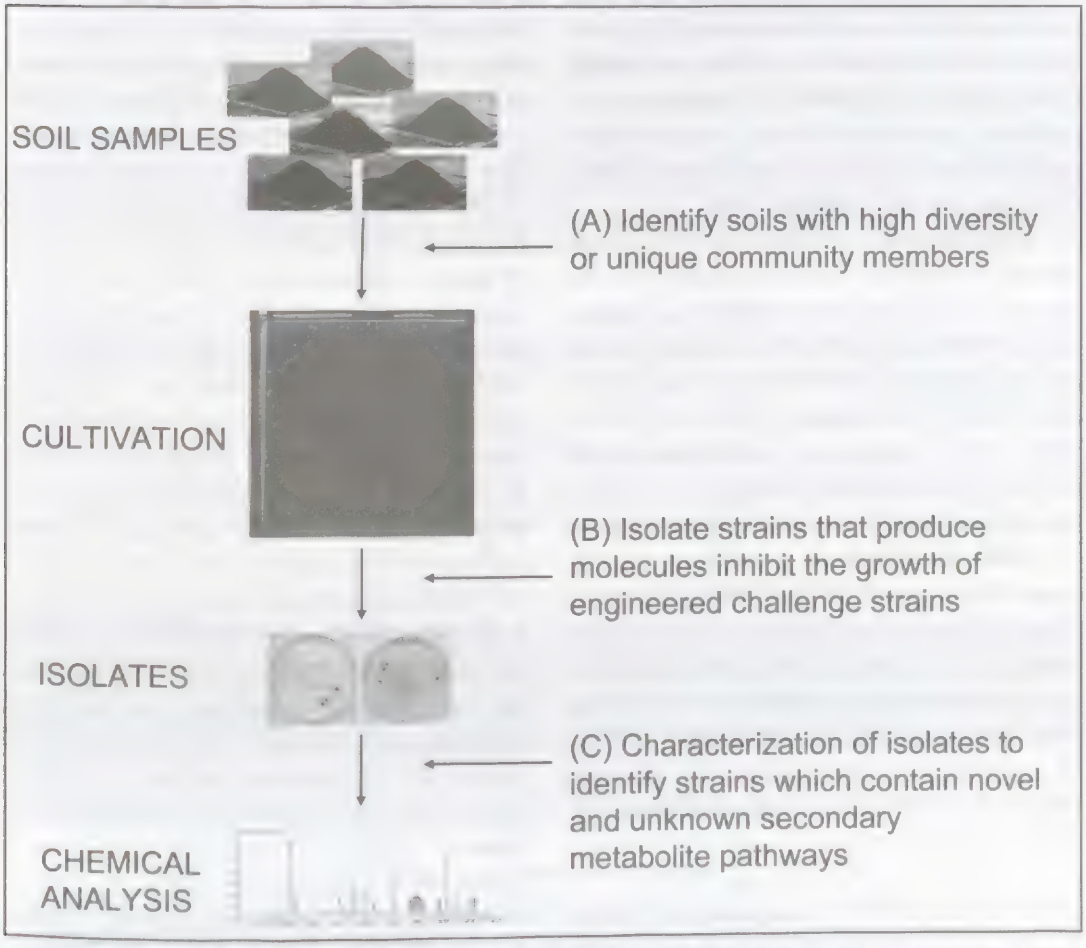
SOIL SAMPLES
(A) Identify soils with high diversity or unique community members
CULTIVATION
(B) Isolate strains that produce molecules inhibit the growth of engineered challenge strains
ISOLATES
(C) Characterization of isolates to identify strains which contain novel and unknown secondary metabolite pathways
CHEMICAL ANALYSIS

Figure 2. The flowchart shows a general procedure for the isolation of actinomycetes from soil. Also shown are points where increases in productivity can be achieved. (A) Soil samples are prescreened for the presence of unique community members. (B) Soil isolates are prescreened by overlay methodologies to identify colonies that produce inhibitory compounds. (C) A genetic profile of the isolate is obtained and compared to a database.

tribution of different lineages, and the authors concluded that geographic isolation and genetic drift were responsible. Similar observations have also been made for bacteria found in soils and in marine sediments. A 16S ribosomal RNA gene survey of 3-chlorobenzoate-degrading bacterial isolates from six regions on five continents revealed that more than 91% of the isolates were unique genotypes that were only found at one site (Fulthorpe, Rhodes, and Tiedje, 1998). More sensitive fingerprinting of fluorescent *Pseudomonas* strains isolated from ten locations on four continents indicated that unique genotypes can be found in different continental regions and that there is no overlap between sites (Cho and Tiedje, 2000). In marine sediments, geographic structuring has been observed for nitrifying, denitrifying, and sulfate-reducing bacteria (Bano and Hollibaugh, 2000; Scala and Kerkhof, 2000; Pérez-Jiménez and Kerkhof, 2005).

Actinomycetes have traditionally been considered more cosmopolitan than other types of bacteria, because they produce highly desiccation- and heat-resistant spores. Their spores provide an efficient mechanism for dispersal and colonization of new environments (Staley and Gosink, 1999). When actinomycete communities found in North American and Central Asian soils were recently compared, however, ample evidence for the presence of endemic populations was detected (Wawrik et al., 2007). Terminal restriction fragment length polymorphism (TRFLP) fingerprinting of 16S ribosomal RNA and type II PKS genes was employed to determine the frequency with which operational taxonomic units (OTUs) are encountered in the environment. Fragment frequencies followed log-normal patterns of distribution, indicating that most actinomycete species and PKS genes are infrequently present in soil. Only 1% of OTUs were detected in more than 50% of samples, and 18% of the OTUs were unique to just one of the 91 soil samples that were analyzed. A strong and significant tendency of the actinomycete 16S rRNA gene fingerprints to cluster by continent indicates that endemic populations of actinomycetes can be found in different regions of the world.

The realization that actinomycete and other bacterial species are composed of endemic populations has significant consequences for bioprospecting. The "everything is everywhere" hypothesis implies that a large majority of the world's actinomycetes is present in only a few samples. If some actinomycetes are endemic, on the other hand, then it is important to identify those soils that contain unique community members. A large number of different methods for the analysis and comparison of microbial communities now exist. The most rigorous approach is to extract DNA from an environmental sample, amplify by means of polymerase chain reaction (PCR) the community of 16S ribosomal RNA genes using universal PCR primers, and to clone the resulting mixture of PCR fragments. Sequence analysis of cloned fragments allows for highly reliable identification of species. Unfortunately, in most cases this approach is impractical and cost prohibitive, especially if a large number of environmental samples need to be analyzed and compared. Fingerprinting technologies such as denaturing gradient gel electrophoresis (DGGE), length heterogeneity PCR (LH-PCR), single-strand conformation polymorphism (SSCP), and TRFLP are instead more commonly used. Each of these methodologies has advantages and drawbacks, but all allow for a direct comparison of community profiles without labor-intensive cultivation or costly sequencing. Fingerprinting technologies have important limitations, especially those inherent in the PCR process. Not all community members are, for example, detected due to primer and amplification biases or because cells are present at a concentration below the limit of detection. A review of PCR-based biases can be found elsewhere (Winzingerode, Gobel, and Stackebrandt, 1997).

The most cost-effective and reliable method for the comparison of microbial communities is TRFLP analysis. Reagent and consumable costs

are less than $5 per sample, and the analysis is adaptable to a 96-well format, which allows for high sample throughput. TRFLP is also highly amenable to computational analysis, because data can be directly exported in digital format from the sequencing machine. TRFLP relies on the separation of different size DNA fragments on a capillary sequencer. Fragments are obtained by endonuclease digestion of community PCR products labeled with a fluorochrome at one end (this is achieved by labeling one of the primers). Different sized, labeled terminal fragments are obtained from different DNA sequences, and each peak in a chromatogram represents at least one (but sometimes more than one) species. The biggest limitation to this analysis is the fact that only 450 different fragment sizes can be separated, and no phylogenetic information can be extracted from the traces. Using multiple enzymes in separate runs can alleviate some of these problems, but makes the analysis computationally more cumbersome. Figure 3 illustrates the use of TRFLP community analysis to compare actinomycete communities. Some peaks (actinomycete species) are detected in many samples (Figure 3A), while others are only present in one soil sample (Figure 3B). Unique and/or unusual communities can in this manner be identified.

TRFLP Analysis of Actinomycete Communities

Many different methods for the extraction of DNA from soil exist. The biggest challenge is removing humic acids that co-purify with DNA

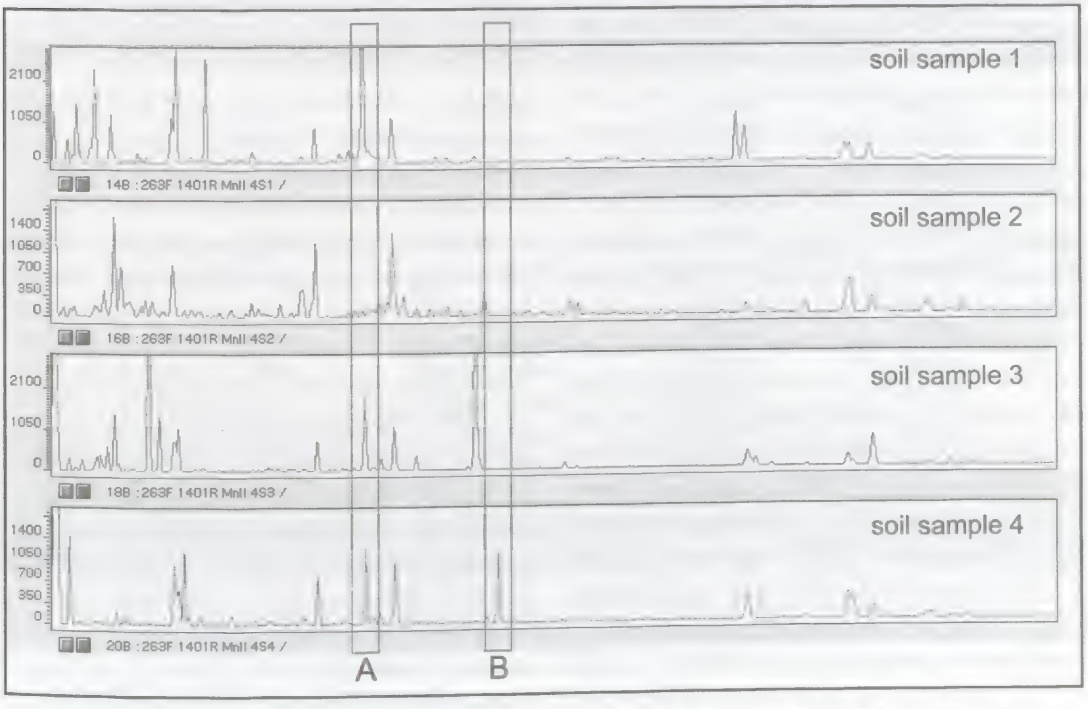

Figure 3. Comparison of actinomycete communities found in soils by means of terminal restriction fragment length polymorphism (TRFLP) analysis. Shown are the fragment patterns obtained from four different soils collected in a forest preserve in New Jersey. While many peaks are found in all four samples, some are only observed in one sample in the data set. (A) Indicates an example of a peak shared by several soil samples. (B) Shows an example of a unique peak. Only soil sample 4 contains a peak at this position, suggesting the presence of a unique type of actinomycete in this soil.

in many protocols. Humic acids will inhibit enzymatic reactions rendering many extracts useless for PCR amplification. Our lab routinely performs two-step purifications. Typically, 0.5 g of soil is extracted using the MO BIO UltraClean Soil DNA kit (MO BIO, Carlsbad, CA) as recommended by the manufacturer. This yields DNA that in most cases will not PCR amplify and needs to be further purified. For further cleanup, the Geneclean Spin Kit (Q-Bio gene, Morgan, Irvine, CA) can be used. In some cases multiple rounds of Geneclean purification are needed. Actinomycete 16S ribosomal RNA genes can be amplified using an actinobacterial group-specific PCR forward primer 243F (17) (5'-GGATGAGCCCGCGGCCTA-3') (Heuer et al., 1997) and the eubacterial reverse primer 1401R (5'-CGGTGTGTACAAGACCC-3'). For TRFLP, the 243F primer is carboxyfluorescein (FAM) labeled. PCR reactions (50 μL) contain 5 ng of template DNA, 200 nM of each PCR primer, 2.5 mM MgCl, 5 U Taq DNA polymerase (Promega, Madison, WI), and 0.4 μL of 10 mg ml^{-1} BSA (Promega). Thirty amplification cycles are run as follows: 1 min at 95°C, 1 min at 60°C, and 1.5 min at 72°C followed by an extension step of 15 min at 72°C. PCR products are then quantified, diluted to 5 ng μL^{-1}, and 30 ng are digested for four hours at 37°C using a restriction endonuclease that cuts a four base pair recognition site. Digests are precipitated using glycogen as a carrier (Wawrik et al., 2005a, 2005b; McGuinness et al., 2006), and the labeled fragments are suspended in 20 μL deionized formamide, denatured at 95°C, and run on an ABI 310 Automated Sequencer (Applied Biosystems, Foster City, CA).

Data analysis is performed by exporting peak data for fragments between 50 and 500 nucleotides in tabular format to Matlab (Natick, MA). Several parameters need to be considered in this analysis, and a discussion can be found elsewhere (Wawrik et al., 2005a, 2005b). Standard analysis is performed by pair-wise comparison of all TRFLP fingerprints in a data set. Typically, a minimum of 50 fluorescence units (FU) is used, and only those peaks accounting for 95% of the area were considered. Most reliable results are obtained by applying a search window of ±0.5 bp to compare all peaks in the data set, and pair-wise matrices are generated by calculating one of several similarity indices (Wawrik et al., 2005a, 2005b). Pair-wise comparisons will yield data on the number of shared and unique peaks for each sample, and similarity values can be used to generate dendrograms. Matlab code for this analysis is available from the authors.

Isolation of Antibiotic-producing Strains

The prescreening of soils for the presence of rich and unusual actinomycete communities by means of TRFLP analysis offers considerable benefits. Ultimately the isolation of bacterial strains in pure culture is required. A typical soil sample contains thousands of different bacterial species (Torsvik, Goksoyr, and Daae, 1990; Torsvik et al., 1998), and it is not unusual to find >100 TRFLP peaks in a single sample analyzed for the diversity of actinomycetes, implying the presence of at least 100 different actinomycete species (and probably many more). Exacerbating the problem is the fact that some actinomycetes are highly ubiquitous. On standard isolation media, *Streptomyces albidoflavus*, for example, is often found to dominate (Vickers, Williams, and Ross, 1984; Williams and Vickers, 1988). Modifying the isolation medium may reduce the proportion of *S. albidoflavus* colonies in favor of other actinomycetes, but in turn biases isolation toward other species (Bull, Ward, and Goodfellow, 2000; Wawrik et al., 2005a). Much has been said in recent years about the need for novel and more general cultivation approaches. No single method has yet proven to be universally applicable, but some have produced results worth mentioning. For oceanic bacteria, for example, extinction dilution methods have been used to capture abundant community members (Button et al., 1993, 1998; Button, 2000). Optical tweezers (Frohlich and Konig, 2000) and gel-microdroplet

encapsulation (Zengler et al., 2002) have also been used to cultivate single cells from environmental samples.

Even with sophisticated isolation technologies only a small number of novel actinomycete strains will probably be found in the backyards of Europe's and North America's large drug companies. Soils close to home have been studied for many decades. Novel diversity is more likely to be recovered from remote regions of the world or from extreme environments. Often such habitats are located inside the territory of developing nations, whose resources cannot provide the financial backing and technology needed for modern bioprospecting operations. Developing economies can, however, offer substantial cost savings in labor-intensive screening endeavors that are no longer economical in the U.S. or Europe. The Central Asia ICBG thus focuses on implementing simple and low-cost screening approaches in partnering third-world countries (in our case Kyrgyzstan and Uzbekistan). Our collaborators in Central Asia can isolate and perform initial screens on a large number of bacterial isolates for a fraction of the cost incurred in the United States. Antibiotic-producing actinomycetes are isolated "in country" by means of traditional overlay techniques. Soil dilutions are plated on large 250 mm^2 Petri dishes and assayed using challenge strains whose growth inhibition (clearing zones) indicates active colonies.

Reducing the rate of rediscovery (e.g., the repeated isolation of strains that produce streptomycin, for example) is a critical challenge to traditional, isolation-based bioprospecting. A simple

				1st screen		2nd screen		3rd screen	
Date	Source ID	Medium	Isolate ID	assay strain	positive	assay strain	positive	assay strain	positive
5/25/2005	KMI-0010A	YEPG	A	*E. coli* JS5 Az	+	pCFF34	-	pMG223	-
5/25/2005	KMI-0010A	YEPG	B	*E. coli* JS5 Az	+	pCFF34	-	pMG223	-
5/25/2005	KMI-0010A	YEPG	C	*E. coli* JS5 Az	+	pCFF34	-	pMG223	-
5/25/2005	KMI-0010A	YEPG	D	*E. coli* JS5 Az	+	pCFF34	-	pMG223	-
5/25/2005	KMI-0010A	YEPG	E	*E. coli* JS5 Az	+	pCFF34	-	pMG223	-
5/25/2005	KMI-0010A	YEPG	F	*E. coli* JS5 Az	+	pCFF34	+	pMG223	+
5/25/2005	KMI-0010A	YEPG	G	*E. coli* JS5 Az	+	pCFF34	+	pMG223	+
5/25/2005	KMI-0010A	YEPG	H	*E. coli* JS5 Az	+	pCFF34	-	pMG223	-
5/25/2005	KMI-0010A	YEPG	I	*E. coli* JS5 Az	+	pCFF34	-	pMG223	-
5/25/2005	KMI-0010A	YEPG	J	*E. coli* JS5 Az	+	pCFF34	-	pMG223	-
5/25/2005	KMI-0010A	YEPG	K	*E. coli* JS5 Az	+	pCFF34	+	pMG223	-
5/25/2005	KMI-0010A	YEPG	L	*E. coli* JS5 Az	+	pCFF34	+	pMG223	-
5/25/2005	KMI-0010A	YEPG	M	*E. coli* JS5 Az	+	pCFF34	+	pMG223	+
5/25/2005	KMI-0010A	YEPG	N	*E. coli* JS5 Az	+	pCFF34	-	pMG223	-
5/25/2005	KMI-0010A	YEPG	O	*E. coli* JS5 Az	+	pCFF34	+	pMG223	+
Total					15		6		4

Table 1. Screening results of actinomycete isolates challenged with multiple antibiotic-resistant *Escherichia coli*. The *E. coli* strain JS5 Az is resistant to presence azide (to prevent contamination), but contains no resistance to antibiotics. Strain pCFF34 (Jacoby and Sutton, 1991) is resistant to Ak, Km, Nm, Su, Tc, and Tm. pMG223 (Jacoby and Sutton, 1991) contains resistances to Ak, Nm, Su, Tc, Tm, Tp, Cm, Gm, Sm, and Sp. (Ak = amikacin; Cm = chloramphenicol; Km = kanamycin; Nm = neomycin; Sm = streptomycin; Sp = spectinomycin; Su = sulfonamide; Tc = tetracycline; Tm = tobramycin; Tp = trimethoprim; Gm = gentamycin.) Both strains make extended-spectrum beta lactamases (ESBLs) active on most beta-lactam antibiotics except carbapenems and cephamycins. *Escherichia coli* is intrinsically resistant to erythromycin and vancomycin.

			1st screen		2nd screen		3rd screen	
Date	Soil sample	colonies	assay strain	positive	assay strain	positive	assay strain	positive
5/13/2005	UMZ-0013	1078	*E.coli* JS5 Az	25	pCFF34	14	pMG223	9
5/13/2005	UMZ-0039	1961	*E.coli* JS5 Az	32	pCFF34	16	pMG223	17
5/13/2005	UMZ-0056	1303	*E.coli* JS5 Az	21	pCFF34	12	pMG223	10
5/8/2005	KMI-002A	1785	*E.coli* JS5 Az	17	pCFF34	12	pMG223	12
5/9/2005	KMI-008D	1911	*E.coli* JS5 Az	9	pCFF34	2	pMG223	2
5/2/2005	KMI-010A	1667	*E.coli* JS5 Az	18	pCFF34	7	pMG223	4
Total		9705		122		63		54

Table 2. Screening results from isolation experiments using soils from Uzbekistan (UMZ) and Kyrgyzstan (KMI). For resistance profiles of the *Escherichia coli* strains JS5 Az, pCFF34, and pMG223, see Table 1.

and cheap methodology that can be implemented in developing countries is the use of challenge strains containing resistances to known classes of antibiotics. The Central Asia ICBG employs *Escherichia coli* strains with resistances to a number of clinically relevant antibiotics of actinomycete origin. Tables 1 and 2 illustrate the use of multiply resistant challenge strains in screening actinomycete isolates. Table 1 shows data from the screening of 15 isolates obtained from Kyrgyz soil. All 15 strains are able to inhibit the growth of *E. coli* containing no antibiotic resistances (first screen), but only six were capable of inhibiting the growth of the multiply resistant *E. coli* strain pCFF34 (second screen). Of these six, only four strains produced zones of inhibition when challenged with strain pMG223 (third screen). Table 2 shows an example of a larger screening experiment using Kyrgyz and Uzbek soil. In this experiment 9,705 colony-forming units (CFUs) were screened, generating 122 isolates that inhibit the growth of non-resistant *E. coli*. Two successive rounds of screening against pCFF34 and pMG223 reduced the number of positive isolates to 54. The remaining 68 isolates likely produce antibiotics that are structurally similar or identical to compounds for which pCFF34 and pMG223 exhibit resistance. The screen hence effectively reduced the rate of rediscovery by 56%.

These data illustrate that even a fairly low-tech screening methodology can reduce the rate of rediscovery by a significant proportion. They also demonstrate that a large proportion of antibiotics producing isolates (>50%) make a relatively small number of different molecules. Extrapolation of these data suggests that it might be possible to reduce the rate of rediscovery by as much as 95% if tester strains containing resistances to only 50 commonly found antibiotics were employed.

Molecular Characterization of Isolates

Strain collections are typically assayed to identify the bioactive secondary metabolites that are produced by individual isolates. Traditionally this involves fermentation in large-batch cultures and subsequent chemical analysis. Such analysis is time consuming, and redundancy at this level is highly wasteful. Colony morphology alone can, unfortunately, not be used to distinguish isolates, because different species of actinomycetes often have very similar colony morphologies (Ritacco et al., 2003). Highly reliable species identification is possible by sequencing the 16S rRNA gene, but even different strains of the same species can sometimes produce a different suite of secondary metabolites. Methods that are capable of dereplicating isolates at the strain level are therefore more desirable (Bull,

Ward, and Goodfellow, 2000; Ritacco et al., 2003). Many approaches for strain differentiation exist, including randomly amplified polymorphic DNA (RAPD) (Anzai, Okuda, and Watanabe, 1994), pulse field gel electrophoresis (PFGE) (Pfaller et al., 1996), ribotyping (Pfaller et al., 1996), and DNA-microarrays (Wen et al., 2006). Most such techniques are very labor intensive (RAPD or PFGE) or are not practical for the purpose of typing novel isolates (microarrays). Other techniques are not useful, because they rely on identifying differences associated with the 16S rRNA operon, which contains no information about secondary metabolism. A better approach is to assay differences in the secondary metabolism gene content of isolates. Particularly promising in this regard is the use of the RiboPrinter microbial characterization system (DuPont Qualicon, Wilmington, DE) as described by Ritacco et al. (2003). In this study, 26 actinomycete isolates were obtained from Hawaiian soils and their rRNA gene sequences, MIDI (Microbial Identification Inc.) fatty acid content, and liquid chromatography-mass spectrometry (LC-MS) profiles were compared to RiboPrinter profiles generated by probing with a non-ribosomal peptide synthase (NRPS) gene. RiboPrinter analysis was able to distinguish between morphologically similar, but genetically distinct, actinomycete isolates containing unique fatty acid and fermentation profiles (Ritacco et al., 2003). The method works by transferring restriction digested genomic DNA, which is separated on an agarose gel, to a membrane. The membrane is then assayed by Southern blot hybridization using labeled DNA probes. The RiboPrinter automates most of the steps, allowing for efficient and rapid analysis of many isolates. In most cases, strain dereplication based on ribotype (16S rRNA gene) is highly efficient and correlates well with metabolite profiles. A great strength of the method also lies in the ability to easily accept other probes. The vast diversity of secondary metabolites produced by bacteria is synthesized by a surprisingly small number of different types of genes. Theoretically, only a small number of gene probes can be used to obtain unique profiles for a large proportion of isolates. Analysis on an automated machine also allows rapid and direct comparison of profiles to a database of past experiments. The major limitation of the technology is probe design. Hybridization experiments generally will not detect gene sequences that are less than 70% identical to the probe. Highly unusual gene sequences may then not be detected in the RiboPrinter profile. Another limitation is the need for custom-made molecular weight markers for each new set of probes. Without these, new prints cannot be normalized or databased by the RiboPrinter system.

Cultivation-independent Bioprospecting

Actinomycetes have been an extraordinarily prolific source of bioactive metabolites. Several lines of evidence suggest, however, that they account for only a small fraction of bacterial diversity in the environment. Initial surveys of 16S ribosomal RNA gene sequences in marine environments demonstrated the presence of many uncultivated lineages (Olsen et al., 1986; Pace et al., 1986). Later, these observations were confirmed for many other environments, including soils. In fact, soils in particular appear to harbor enormously diverse bacterial populations (Borneman and Triplett, 1997; Hugenholtz, Goebel, and Pace, 1998). Evidence from reassociation kinetics experiments suggests that even a single soil sample can contain thousands of different bacterial genomes (Torsvik, Goksoyr, and Daae, 1990; Torsvik et al., 1996, 1998), potentially making uncultured soil bacteria the largest reservoir of genetic information on the planet (Wawrik et al., 2005a). It is now commonly believed that 99% of bacterial diversity is not captured in culture collections. The potential for novel biochemistry and secondary metabolism in this uncaptured majority surpasses that of combinatorial chemistry approaches (Bull, Ward, and Goodfellow, 2000). A relatively nascent field of microbiology that considers the challenges of ex-

ploiting novel biocatalytic activities of uncultivable bacteria is "metagenomics." The basic premise of metagenomics is the fact that DNA of unculturable bacteria can be isolated and then cloned into other hosts such as *Escherichia coli*, where it can be studied. The "metagenome" is the mixed assembly of different genomic fragments found in a library of randomly cloned environmental DNA. A large enough metagenomic library, in theory, should be able to capture the entire genomic content of all bacteria in a sample. Metagenomics is a particularly exciting and new frontier of natural products discovery and promises to invigorate interest in microbial bioprospecting.

Metagenomics and Microbial Ecology

Initial work in the area of metagenomics occurred primarily in the field of microbial ecology. Stein et al. (1996) pioneered the approach by cloning bulk North Pacific marine picoplankton community DNA into a fosmid vector. The library was screened for the presence of archaeal rRNA gene sequences closely related to planktonic crenarchaeota, and a 38 kb clone was identified. Several other archaeal genes were identified, including elongation factor 2 (EF2) and two genes not previously observed in archaea (RNA helicase and glutamate semialdehyde-aminotransferase). A similar approach was applied by Béjà et al. (2000b), who constructed large insert BAC (bacterial artificial chromosome) libraries from marine plankton, with average insert sizes of 80 kb (as much as 150 kb). Screening of the library revealed that its phylogenetic composition was in good agreement with PCR-based 16S ribosomal RNA gene surveys. A significant number of clones originated from uncultured microbes, and one euryarchaeotal clone was sequenced in its entirety, revealing several previously unknown archaeal genes. During the same study, a pigment-producing BAC clone was noticed. Further analysis of this clone identified a type of bacteriorhodopsin (Béjà et al., 2000a). Bacteriorhodopsins typically function as light-driven proton pumps in certain halophilic bacteria and had not previously been described for marine pelagic bacterioplankton. The pigment was shown to be associated with an uncultivated gamma-proteobacterium and shared sequence similarity with archaeal rhodopsins. Expression of the gene in *Escherichia coli* produced a functional proton pump with typical photochemical properties. In another study, BAC libraries were used to study niche partitioning, metabolic variability, and population structure of marine picoplankton communities found in surface water (Suzuki et al., 2004). Libraries were screened for the presence of 35 phylogenetic groups using variations in the length of the internal transcribed spacer (ITS) region of the large subunit 16S rRNA gene. Direct cloning of environmental DNA allows for more accurate description of microbial communities, because the method is free of amplification biases introduced by PCR. It also allows for sequencing of complete ribosomal RNA genes (beyond the sites of the universal primers commonly used), enabling the authors to refine phylogenetic analyses of planktonic marine alpha and gamma proteobacteria in Monterey Bay. These and many subsequent studies have demonstrated that metagenomic analyses can provide profound insights into the ecology, biochemistry, and metabolism of uncultured microbes in the environment.

Metagenomic Surveys of Soil Systems

Metagenomic bioprospecting of the immense diversity of uncultivated bacteria in soil systems creates exciting opportunities, but faces unique challenges. Primarily, it is difficult to obtain quality high molecular weight (HMW) DNA from soil. Commonly used column purification systems reduce humic acid contamination, but shear DNA into small fragments that are not useful for metagenomic cloning. The first application of the technology to soil systems, therefore, did not ap-

pear in the literature until several years after initial work in marine environments. A BAC vector was used to construct soil metagenomic libraries containing more than one gigabase of cloned DNA fragments (Rondon et al., 2000). When the phylogenetic diversity of the cloned DNA was investigated using 16S rRNA gene sequences, it was found that the libraries contained DNA from at least four different bacterial phyla, including low-G+C gram positives, *Acidobacterium*, proteobacteria, and *Cytophagales*. Ribosomal RNA genes from many other bacterial groups were identified in a later, more thorough survey (Liles et al., 2003), indicating that DNA from a wide diversity of bacteria can be cloned from soil. The same study, however, also points out several discrepancies between the BAC library content and PCR-based surveys of the same soil communities. The BAC library contained a lower frequency of *Bacillus*, α-proteobacterial, and *Cytophaga–Flavobacterium–Bacteroides*-group 16S signatures, and no archaeal sequences, pointing to methodological biases that might skew the phylogenetic composition of metagenomic libraries and could limit the ability to clone DNA from all groups of uncultivated bacteria.

An exciting observation that emerged from these studies is the fact that many clones express enzymatic activity heterologously in *Escherichia coli*. Heterologous expression in *E. coli* is not necessarily expected, because many soil bacteria are only distantly related to *E. coli*. Clones producing lipase, amylase, nuclease, and hemolysis activity were nonetheless detected in the study by Rondon et al. (2000). When genomic DNA from *Bacillus cereus* (Gram +) was cloned into a BAC vector and transformed into *E. coli* (Gram −), it was found that six out of ten *B. cereus* traits that were assayed were expressed in *E. coli* (expressed traits were ampicillin and zwittermicin A resistance, esculin hydrolysis, hemolysis, orange pigment production, and lecithinase activity). These data indicate that in many cases it is possible to directly assay clones for activity, making the screening of libraries extremely cost effective. Several studies have subsequently adopted different activity-based approaches. Henne et al. (2000) constructed a metagenomic library by cloning 5 kb– to 8 kb–sized fragments of environmental DNA into pBluescript SK(+) and transforming the plasmid into *E. coli* DH5α. The library was screened for lipolytic activity by directly plating transformants onto two different indicator medium types that allowed for direct visual identification. A total of four active colonies were detected at a frequency of 1.4×10^{-6} and 3.5×10^{-6} per colony for the assays, respectively. The amino acid sequences for the cloned proteins were only 34% to 48% identical to sequences found in public databases, indicating that highly novel forms of known genes can be isolated from metagenomic libraries. In a very similar study by the same group (Henne et al., 1999), five recombinant plasmids among 930,000 (insert sizes of 5–8 kb) containing metagenomic clones were identified, which allowed *E. coli* to grow on 4-hydroxybutyrate as a sole energy and carbon source by expressing 4-hydroxybutyrate dehydrogenase activity. A screen of 900,000 clones on indicator agar identified 15 transformants capable of converting short-chain polyols (1,2-ethanediol, 2,3-butanediol, and a mixture of glycerol and 1,2-propanediol) into corresponding carbonyl compounds (Knietsch et al., 2003). Seven of the clones also exhibited NAD(H)-dependent alcohol oxidoreductase activity in crude extracts. Most of the genes responsible for activity were similar to genes of unknown functions or not similar to any gene in the database, indicating that novel classes of genes for relevant biochemical transformations can be identified and isolated by direct screening of metagenomic libraries.

Direct screening of metagenomic libraries has been successfully applied in order to identify clones with antimicrobial activity. The first successful isolation of a novel antimicrobial secondary metabolite from a metagenomic source was a survey of 700,000 cosmid clones (in *Escherichia*

coli), which were screened by soft agar overlay against *Bacillus subtilis* for antimicrobial activity (Brady and Clardy, 2000). Sixty-five colonies produced clearing zones and were grown and extracted using ethyl acetate. This resulted in the identification of one highly active extract that was shown to contain a previously unknown long-chain N-acyl-L-tyrosine antibiotic. Sequence analysis revealed the corresponding long-chain N-acyl amino acid synthase (a novel class of secondary metabolite genes). Occasionally, screening is as easily achieved as visual inspection. At very low frequencies colored colonies may be observed when libraries are plated. A blue-pigmented colony was, for example, isolated from a metagenomic soil library and shown to produce two secondary metabolites: deoxyviolacein and the broad-spectrum antibiotic violacein (Brady et al., 2001). Sequence analysis revealed a 6.7 kb gene cluster responsible for violacein and deoxyviolacein production. In another study, a fosmid library was constructed from Jindong Valley (Korea) forest soil and observed to contain several colonies that produced purple or brown pigments (Lim et al., 2005). One of the colored colonies also exhibited antimicrobial activity against *B. subtilis* and was shown to produce the pigments indirubin and indigo blue. Production of the pigments was linked to the expression of two open reading frames (ORF) that encode for an oxygenase-like protein and a putative transcriptional regulator. Similarly, inspection of a metagenomic soil library by Gillespie et al. (2002) revealed three colonies that produced a brown melanin-like color. Methanol extraction of one of the clones simultaneously produced a red and an orange pigment, both of which were shown to be triaryl cations and designated turbomycin A and B, respectively. Both were shown to exhibit broad-spectrum antibiotic activity against gram-negative and gram-positive bacteria. Sequence analysis identified a single open reading frame similar to the 4-hydroxyphenylpyruvate dioxygenase (4HPPD) family of enzymes responsible for the production of the pigments. Turbomycin production was complemented by genes intrinsic to *E. coli* showing that chimeric pathways can be created from genes cloned from the environment leading to the production of novel chemical structures.

Heterologous expression of cloned genes can be achieved in hosts other than *Escherichia coli*. This may be desirable, because a large majority of known bacterial secondary metabolites are produced by gram-positive bacteria (mostly *Streptomyces*). Using a streptomycete host for generating metagenome libraries should enhance the likelihood of expressing genes that might otherwise fail to be expressed in a gram-negative host. An *E. coli* to *S. lividans* shuttle vector has been constructed by inserting the pSAM2 *Streptomyces* integrative element into the *E. coli*–based cosmid pWED1 (Courtois et al., 2003). Libraries were generated in *S. lividans* and *E. coli*, and a total of 1,700 and 800 colonies, respectively, were screened directly by HPLC analysis. HPLC profiles of two clones in *S. lividans* contained peaks not detected in the host strain. The peaks were shown to correspond to a fatty dienic alcohol ester not previously described in the literature.

Despite such successes, there are limitations to the usefulness of a streptomycete-cloning host. It favors the expression of actinomycete genes, biasing the screen toward genes from these already highly studied and eminently culturable bacteria. It is unlikely that culture collections accurately reflect the distribution of secondary metabolite genes in bacteria as a whole. Direct expression in libraries may not always be desirable or even possible. Pathways for the production of antibiotics, in particular, can be toxic to their host unless an appropriate resistance mechanism is also cloned. Proper precursor molecules might not be synthesized by the host, even if genes are expressed. Many secondary metabolite biosynthetic pathways are also very large, encompassing tens of thousands of base pairs, making it unlikely that complete and functional pathways are captured.

A more direct approach to identifying clones that contain secondary metabolite gene clusters is the use of molecular tools (Figure 4). The more commonly used technique is the filter array. Thousands of colonies can be spaced very tightly onto a membrane using a colony-picking robot. The filter is placed onto agar medium to allow colony growth and is then lifted. Cells are lysed by placing the filter onto paper soaked with lysis solution. After lysis, the filters are microwaved and subsequently digested with proteinase K to remove cellular debris. DNA is crosslinked to the filters and can then be probed using different methodologies (Figure 4A). Colonies are spotted in duplicate, and the unique orientation of duplicate spots can be used to identify individual microtiter plates, while the relative position of spots reveals the row and column that contain the targeted clone. Probes are typically generated by PCR amplification from the DNA used in library construction or from a cloned gene. An alternate methodology is to screen pooled plasmid DNA from each microtiter plate by PCR (Figure 4B). Pooled DNA is generated by replicating microtiter plates onto petri dishes, collecting the cells from the plates after colonies have grown, and extracting plasmid from the pooled cell mass. Even with only a modest number of PCR reactions, a very large number of clones can efficiently be screened in this manner. PCR targeting different classes of secondary metabolite genes can be performed using a suite of primer sets. Several studies are found in the literature that have aimed at developing broad-range degenerate PCR primer sets for the detection of secondary metabolite gene clusters (Table 3). Once a microtiter plate has been identified to contain a targeted gene, pooled DNA is generated for each row and each column of that plate. These pools are then further screened by PCR to identify the relevant clone (Figure 4C). Clearly the major limitation to these methodologies is primer and probe design. PCR primers can only be based on known sequence information. Sequence data-

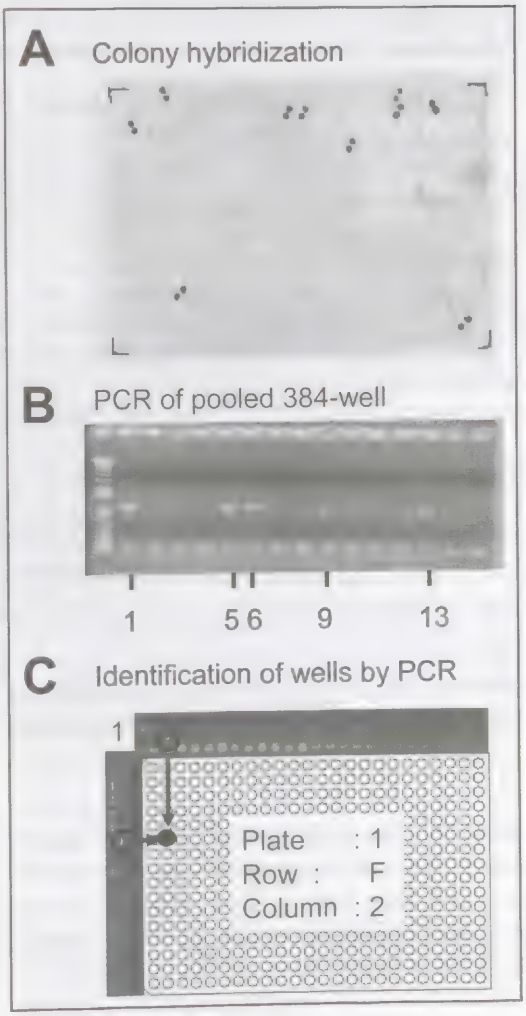

Figure 4. Illustration of methods used to identify secondary metabolite genes in metagenomic libraries. (A) Filter hybridization: 3,000 BAC clones were arrayed in duplicate onto a membrane, which was probed using a cloned gene. The unique orientation of duplicate spots can be used to identify individual plates. Their relative position on the filter indicates the row and column containing a clone that produces hybridization signal. (B) PCR of pooled plasmid DNA: Plasmid was extracted from pooled cell pellets generated from each 384-well plate. DNA from each pool was PCR amplified using *Actinomyces*-specific primers. This screen identifies microtiter plates containing clones of interest (here plates 1, 5, 6, 9, and 13). (C) Rows and columns of individual, positive 384-well plates are pooled, and PCR is used to identify wells.

Secondary Metabolite Gene	Primers sequences	Reference
Type I PKS	5'-TSAAGTCSAACATCGGBCA-3' 5'-CGCAGGTTSCSGTACCAGTA-3'	(Ayuso-Sacido and Genilloud, 2005)
"	5'-CCSCAGSAGCGCSTSYTSCTSGA-3' 5'-GTSCCSGTSCCGTGSGYSTCSA-3'	(Ginolhac et al., 2004)
"	5'-MGNGARGCNNWNSMNATGGAYCCNCARCANMG-3' 5'-GGRTCNCCNARNSWNGTNCCNGTNCCRTG-3'	(Piel, 2002)
"	5'-CCSCAGSAGCGCSTSTTSCTSGA-3' 5'-GTSCCSGTSCCGTGSGTSTCSA-3'	(Courtois et al., 2003)
"	5'-CCSCAGSAGCGCSTSCTSCTSGA-3' 5'-GTSCCSGTSCCGTGSGCCTCSA-3'	(Courtois et al., 2003)
Type II PKS	5'-TSG CST GCT TGG AYG CSA TC-3' 5'-TGG AANCCG CCG AAB CCG CT-3'	(Metsä-Ketelä et al., 1999)
"	5'-GGITGCACSTCIGGIMTSGAC-3' 5'-CCGATSGCICCSAGIGAGTG-3'	(Wawrik et al., 2005b)
"	5'-TTCGGCGGITTCCAGTCIGCCATG-3' 5'-TCCAGCAGCGCCAICGACTCGTAICC -3'	(Seow et al., 1997)
NRPS	5'-GCSTACSYSATSTACACSTCSGG-3' 5'-SASGTCVCCSGTSCGGTAS-3'	(Ayuso-Sacido and Genilloud, 2005)

Table 3. Degenerate PCR primers used to detect secondary metabolite genes.

bases are still comparatively small, and any PCR primer set will be biased in favor of sequences similar to those already known. Truly novel gene sequences are likely to be very different from those found in the database. Probing filters suffer from the same limitations as the RiboPrinter technology discussed above. The technology is limited to detecting sequences more than 70% identical to the sequence of probes used in detection.

Concluding Remarks

Actinomycetes have been a tremendously prolific source of bioactive metabolites. Despite the decline in the number of novel small molecules being described from this group today, there is good reason to be optimistic about the future of microbial bioprospecting as a source of medically and industrially important biochemistry. The tools of microbial ecology can be used to identify unique communities containing novel strains. They can also be used to discern isolates, helping to reduce redundant analysis and improve productivity. More importantly, molecular tools promise access to the genetic material of yet-to-be-cultured microbes in the environment. Even the relatively modest number of metagenomic studies found in the literature have already identified an impressive number of novel metabolites. As the technology matures, it will be possible to handle larger libraries and perform more sophisticated screens. The greatest genetic resource on earth is made available to bioprospecting efforts by the tools of metagenomics. Soil may yet again prove to be an exciting place for discovery as the genetic secrets of the vast majority of uncultivated bacteria are unlocked.

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Modern Innovations in Natural Products Research

Genomic Tools for the Discovery of Natural Products: What Have We Missed?

James B. McAlpine[1]

The biosynthetic pathways that lead to the vast array of secondary metabolites have been studied for over 50 years. Initial conjecture, on the basis of structural similarities, gave way to radioactive precursor incorporation studies, enzyme isolation, stable isotope incorporation, and, most recently, gene expression. This last subject has proved to be a gold mine, particularly in the study of bacteria, where it has been found that virtually all of the genes, encoding the enzymes used in the assembly of even a quite complex secondary metabolite, are clustered in a single locus on the chromosome. Moreover, the modular nature of many of these biosynthetic systems, and the correspondence between the arrangement of genes and the order in which the enzymes are used in the biosynthesis, allows for a prediction of the final structure, or partial structure, from the gene sequence. Thallion Pharmaceuticals has built this genomic science into a drug discovery platform. A database correlating gene sequence to metabolite (sub)structure has been developed, with more than 65,000 genes clustered in more than 1,600 loci. As gene sequencing has become more facile, it is now possible to sequence the genome of an actinomycete in a couple of days. The IT tools have been assembled to deconvolute that sequence and determine the biosynthetic capability of the organism. Similarly, programs have been written to assist in the detection and thereby the isolation of any chosen metabolite. It is now apparent that almost any strain of streptomyces has the ability to produce more than a dozen, some strains more than 30, different secondary metabolite families. It has long been recognized that different strains of the same species have very different biosynthetic capabilities. Hence, despite 60 years of intense industrial and academic research in this area, only a small percentage of the secondary metabolic capacity of the actinomycetes has been mined.

Introduction

Over the last 20 years the understanding of the biosynthesis of antibiotics and other complex secondary metabolites has gone from the precursor incorporation/enzymatic studies to the gene level. Thallion has built a drug discovery platform based on these genomic advances and has developed bio-informatic tools, which translate the gene sequence into the chemical structures that the microbe is capable of producing. Further chemo-informatic programs guide the identification, isolation, and purification of a chosen metabolite. Applications of this platform provide evidence for the underutilization of well-studied microbes and emphasize the degree to which natural products are undiscovered.

Discussion: 1. Genomic Tools

Actinomycetes have proved to be a very valuable source of a wide variety of medicinally important drugs. These encompass many different structural types and biological activities, from antibacterials to herbicides. Secondary metabolites from this source that have become successful drugs themselves include the 14-membered macrolide erythromycin, the glycopeptide vancomycin (both antibacterials), the anticancer drugs mitomycin and daunorubicin, the immunolides rapamycin and tacrolimus, and the polyene antifungal amphotericin B. Although no β-lactam from actinomycetes became a successful drug per se, the cephamycins, a subclass found only in *Actinomyces*, are represented by the semisynthetic anti-anaerobe cefoxitin. Actinomycetes

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are filamentous bacteria. There are about 4,000 species described in the literature with an estimated probable number of 35,000 to 50,000 species existing in nature. Because they are prokaryotes, it is straightforward, in the majority of the cases, to analyze their genome, as there are no intron-exon issues. They have one single, though relatively large, chromosome (from 4 to 12 megabase [Mb]) that can accommodate many gene clusters for the biosynthesis of a variety of secondary metabolites. A typical streptomycete has >20 such loci. Their physiology and molecular genetics are well understood, making them amenable to industrial-scale processes and yield improvement. In the vast majority of cases, all the genes for the many enzymes that assemble each of the secondary metabolites are clustered into a single locus on the chromosome.

In the classical approach for discovery of novel compounds, actinomycetes or other microbes have been grown under a variety of fermentation conditions and their culture extracts screened for biological activity. When an activity was encountered it would be used to guide purification of the active metabolite. Many of the drugs in use today have been discovered through this approach; however, the efficiency of this process for the discovery of new chemical entities is less than 10%, particularly after many years of screening programs. Even with good dereplication systems, using this classical system, ~90% of the structures isolated are known compounds. Thallion has developed a new platform for drug discovery, based on our understanding of the genetics behind the biosynthesis of secondary metabolites. In this approach, Bio/Cheminformatic tools have been developed (and linked to automated DNA sequencing) for analyzing the genome of actinomycetes to identify loci that encode the enzymes for the biosynthesis of secondary metabolites. The gene sequence information is interpreted to predict the chemical structures of the products of the enzymes encoded by each locus. This structure is then assessed for novelty and the presence of any known pharmacophores. The predicted physicochemical properties of the metabolite are used to guide the fermentation, screening, and purification of the compound. The usual structural elucidation by spectral studies is reduced to a confirmation of the predicted structure.

This approach is possible because the genes for the biosynthesis of secondary metabolites are clustered together and, in many cases, have modular organization defining the nature and sequence of the small primary metabolites that are assembled into the larger and more complex secondary metabolites. Thallion's proprietary genome scanning technique (Zazopoulos et al., 2003) allows for detection of all (with 99% probability) biosynthetic loci that may exist in an 8–9 Mb genome by analyzing 0.7–0.8 Mb DNA sequences. This technique enabled assembly of the database economically, both with respect to time and resources. The DECIPHER database was assembled by comparison of gene sequences from different biosynthetic loci. In Figure 1, the orange open reading frames (ORFs) in the vancomycin biosynthetic locus are highly homologous to those in the locus for nocardicin (though not necessarily in the same orientation), and both encode the enzymes leading to p-hydroxyphenylglycine. The red ORFs in the anthramycin and the lincomycin loci encode the enzymes necessary to convert tyrosine into the n-propylprolyl moieties. The blue ORFs in the anthramycin and actinomycin loci are responsible for the 3-hydroxy-4-methylanthranilic acid substructures.

The DECIPHER database contains more than 65,000 gene sequences grouped in more than 1,660 loci with more than half proprietary to Thallion. Recently, the number of complete bacterial genome sequences available in the literature has been growing at a logarithmic rate (Figure 2), with a doubling time of about 20 months. As of September 2006, there were over 400 bacterial strains for which these had been published. Thallion's DecipherIT tools[1] have been adapted to handle these

[1]DecipherIT is the registered trademark for the progams that mine the DECIPHER database, detect loci, annotate the genes, generate partial structures, and present the data in an easily accessible fashion.

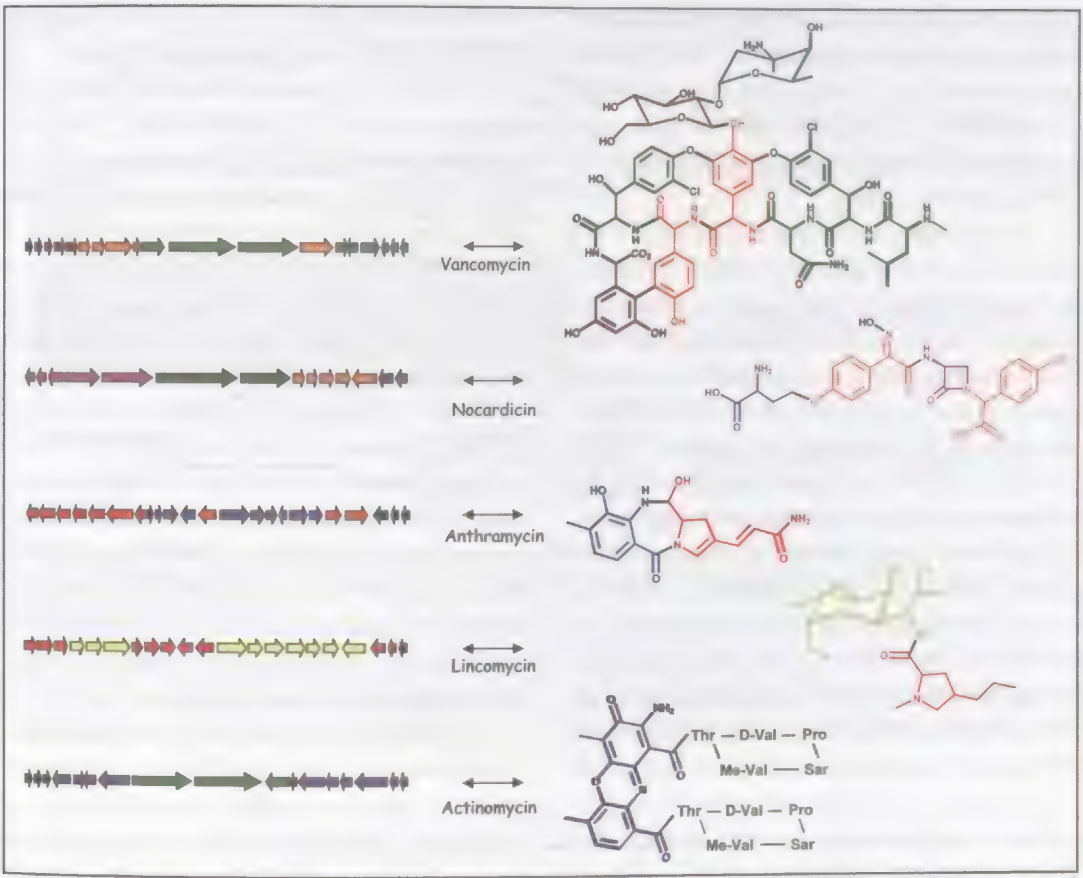

Figure 1. A schematic outline of the genes in the biosynthetic loci leading to antibiotics with a variety of structures but with some common substructures. The color of a depicted gene coincides with the substructure derived from that gene.

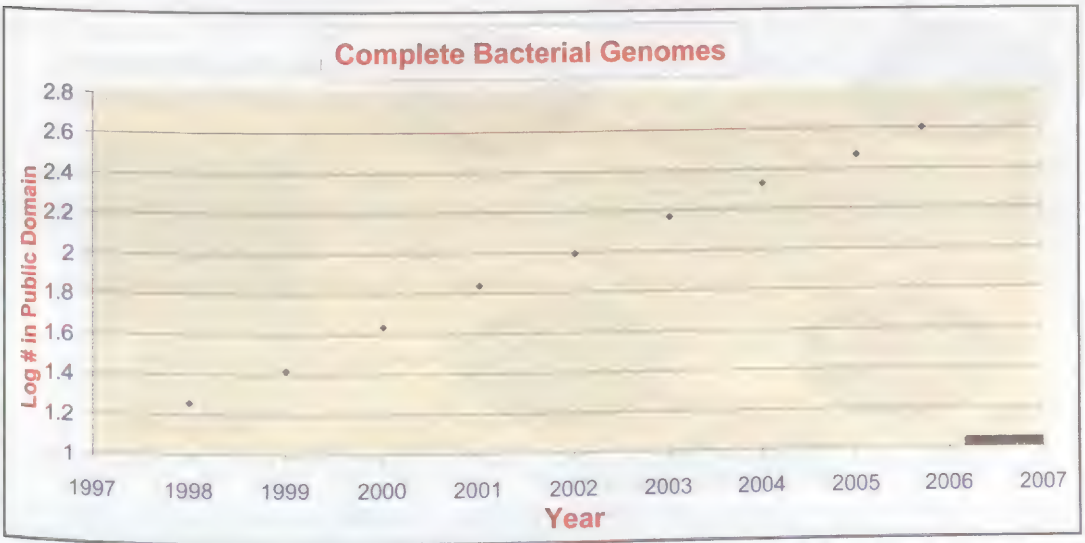

Figure 2. A semi-log plot of the number of complete bacterial genomes appearing in the public literature.

data, and with this software it was possible to detect and rapidly define the products of 33 loci in *Streptomyces avermitilis* and 23 loci in *S. coelicolor,* compared with 30 and 21, respectively, found by the original investigators (Ōmura et al., 2001; Bentley et al., 2002; Ikeda et al., 2003).

A schematic presentation of the DECIPHER genome-based drug discovery system is presented in Figure 3. Either through genome scanning or complete genome analysis, the sequence information is obtained, stored, and analyzed for novel biosynthetic gene loci. The loci are annotated, and the structures of the secondary metabolites, the end-products of their expression, are deduced. The scientist can now prioritize these according to project goals and, on the basis of the structures of the priority compounds, the fermentation conditions and the identification and isolation conditions can be tailored. The structure of the pure product can be rapidly confirmed by classical spectral methods. The compound is then biologically profiled, and a Drug Lead Package is created that includes physico-chemical properties, in vitro activity spectrum, MTD (mice), PK (rats), and in vivo efficacy.

A classic example of the power of the platform is presented by an examination of the vancomycin producer, a strain that has been studied for more than 40 years. Genome scanning of the ATCC-deposited culture, followed by DecipherIT analysis of the sequences, showed, in addition to the gene cluster for vancomycin biosynthesis, 11 other biosynthetic loci–encoding polyketide synthases, non-ribosomal peptide synthases, and an enediyne synthase. We chose to produce and isolate the products of two of these loci; one locus was clustered around type I polyketide synthase (PKS-I) genes and the other around non-ribosomal peptides synthetase (NRPS) genes. Both PKS-Is and NRPSs are large poly-ORF complexes that are modular, and the structural organization allows for prediction of the final product to a high degree of detail and accuracy.

The PKS-I is in a 170+ kb locus that includes several auxiliary genes. Immediately upstream of

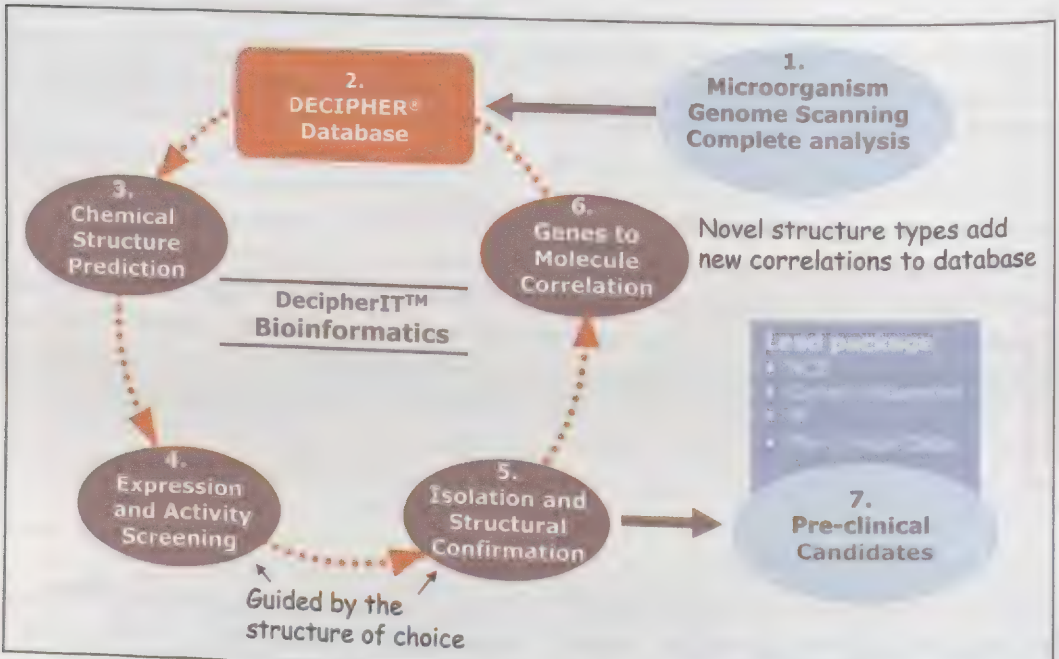

Figure 3. A schematic description of the DECIPHER technology and the steps involved in drug discovery via this approach.

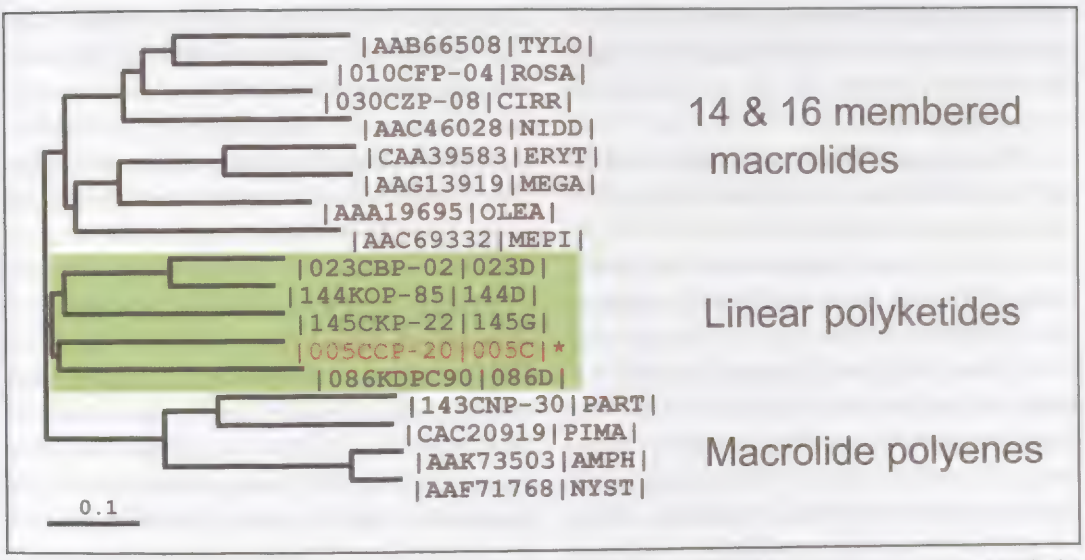

Figure 4. A relationship map of the sequences of the active sites of PKS-I thioesterases correlates with the product structure type.

the PKS are the genes leading to the production of the C5N unit, 2-amino-3-hydroxycyclopent-2-eneone (glycine and succinyl-CoA are converted into aminolevulinate, which is cyclized). Flanking the PKS are genes that will encode the enzymes to convert arginine to guanidinobutryryl-CoA and to transfer this group to the initial acyl-carrier protein of the PKS. Other genes encode an oxidase, which would convert glucose to glucuronic acid, and a glycosyl transferase, which would attach the glucuronic acid as a glucuronide. An N-methyl transferase is the product of a small upstream gene.

Comparative analyses can be made at either the gene level or the protein level. PKS-Is are terminated by a thioesterase, and a comparative analysis of thioesterases from several PKS-Is leads to the family tree shown in Figure 4. Inspection of the tree indicates that this polyketide structure will be linear. The domains immediately before the terminus indicate a pentaene chromophore. The acyltransferases (AT) of the PKS-I define the extent and position of branching.

In this case, the structure of the metabolite, which we have named ECO-0501, is uniquely defined (as shown in Figure 5) by our understanding of the gene sequence data. It must be conceded that the uniqueness of the predicted structure is somewhat fortuitous. The β-keto processing domains of the PKS-I leave only one hydroxyl group, which must be the site of glycosylation, and had

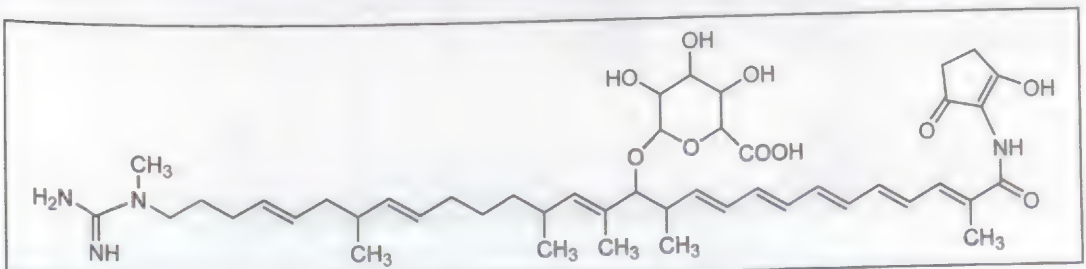

Figure 5. The structure of ECO-0501 as predicted by in silico analysis and confirmed after isolation 5.

there been multiple hydroxyls, our state of knowledge of the specificity determinants for the position of glycosyl transfer would not have allowed for a unique designation.

The best condition for expression of the locus is not well understood, and multiple conditions were employed. A comprehensive extract was prepared and a high-performance liquid chromatography (HPLC) run under standardized conditions with ultraviolet (UV) and positive and negative ion mass spectral detection. Eighty fractions were collected and analyzed for bioactivity. All data were collected on the server and were available for easy comparison between different fermentation conditions. An interactive computer application collects and processes data from the liquid chromatography-mass spectrometry (LC-MS) and UV analyses and presents a graphical interface for locating and identifying the target compound. In Figure 6, each bolded column portrays a different fermentation condition, while fine columns are used for up to four chosen bioassay results. Rows represent HPLC fractions. The chromatographic data are overlaid with bioactivity data. The interactive screen allows the scientist to select the MS output to show only the molecular ion of interest. Here, this slide shows the selection for a positive ion in the range $837 < m/z < 839$ and a negative ion in the range $835 < m/z < 837$, the expected result for the compound sought. Note that a positive ion in this

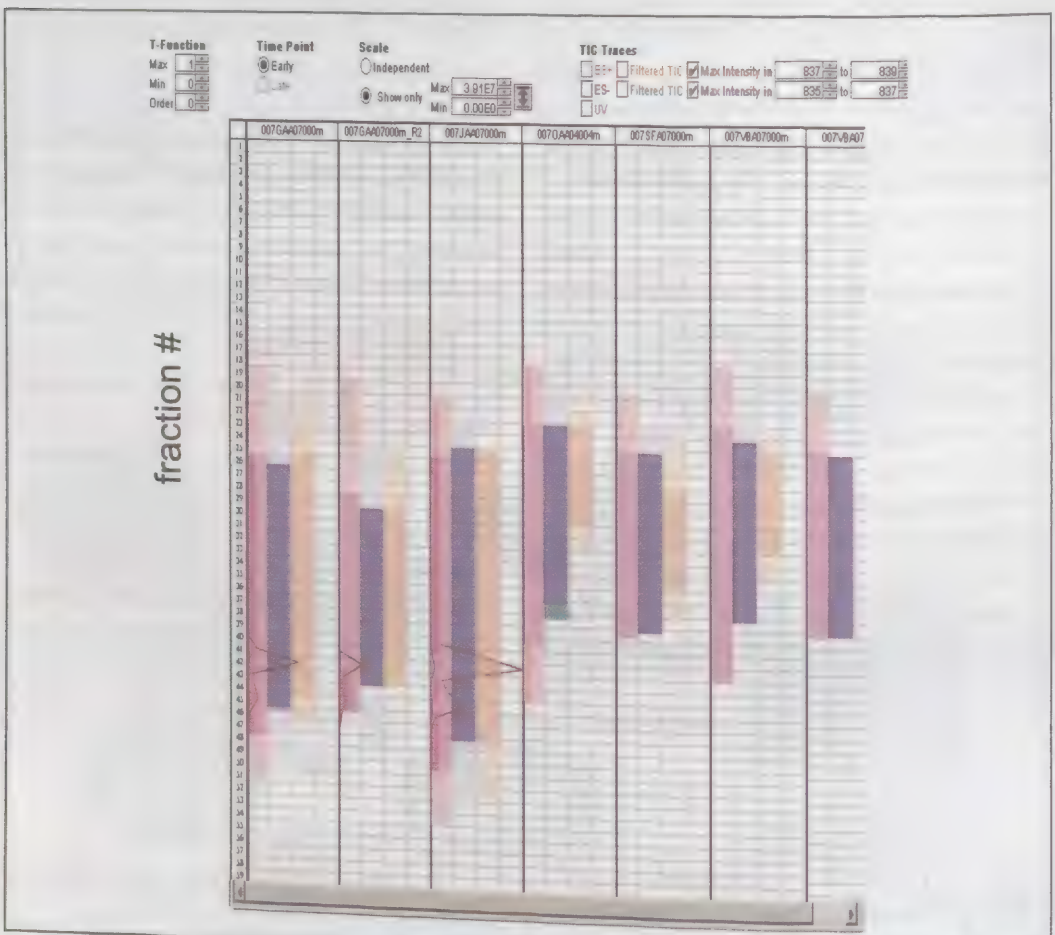

Figure 6. Depiction of one of the IT tools for analysing the HPLC/UV/MS/bioactivity data from multiple fermentations of *Amycolatopsis orientalis* ATCC 43491.

region is only seen in the first three media and a weak negative ion only in the third medium. In each case, the peak defined in this range centered on fraction 41. In this interactive program, the scientist can position the cursor over fraction 41 in the third column and right click the mouse to produce the screen shown in Figure 7, which displays the total composite chemical data for this particular HPLC fraction. The column chosen is displayed at the top of the screen and the composite spectral data (positive-ion MS, negative-ion MS, and UV spectrum) below. The intensity of major signals is given on the right. The blue fraction bar can be selected and moved to another fraction, and all of the spectral data will change to that of the corresponding new fraction selected. Thus, the scientist can be confident the MS and UV data correspond to the same ingredient. The UV spectrum is somewhat poorly resolved but nonetheless definitively characteristic of a conjugated pentaene. With this identification, it is mundane to scale up the fermentation and isolate the ECO-0501, the structure of which is confirmed by high-resolution mass spectra and full 1D and 2D nuclear magnetic resonance (NMR) analyses defining all the H and C parameters (chemical shifts, homo- and hetero-nuclear couplings, and nuclear Overhauser effects).

ECO-0501 represents a novel antibacterial pharmacophore. It is active at 2 μg/mL against several antibiotic-resistant *Staphylococcus aureus* tested as well as vancomycin-resistant *Enterococcus* (VRE) at 4–6 μg/mL. Despite its size, it acts on the plasma membrane in a manner similar to the large cationic peptides, such as melittin. It is very

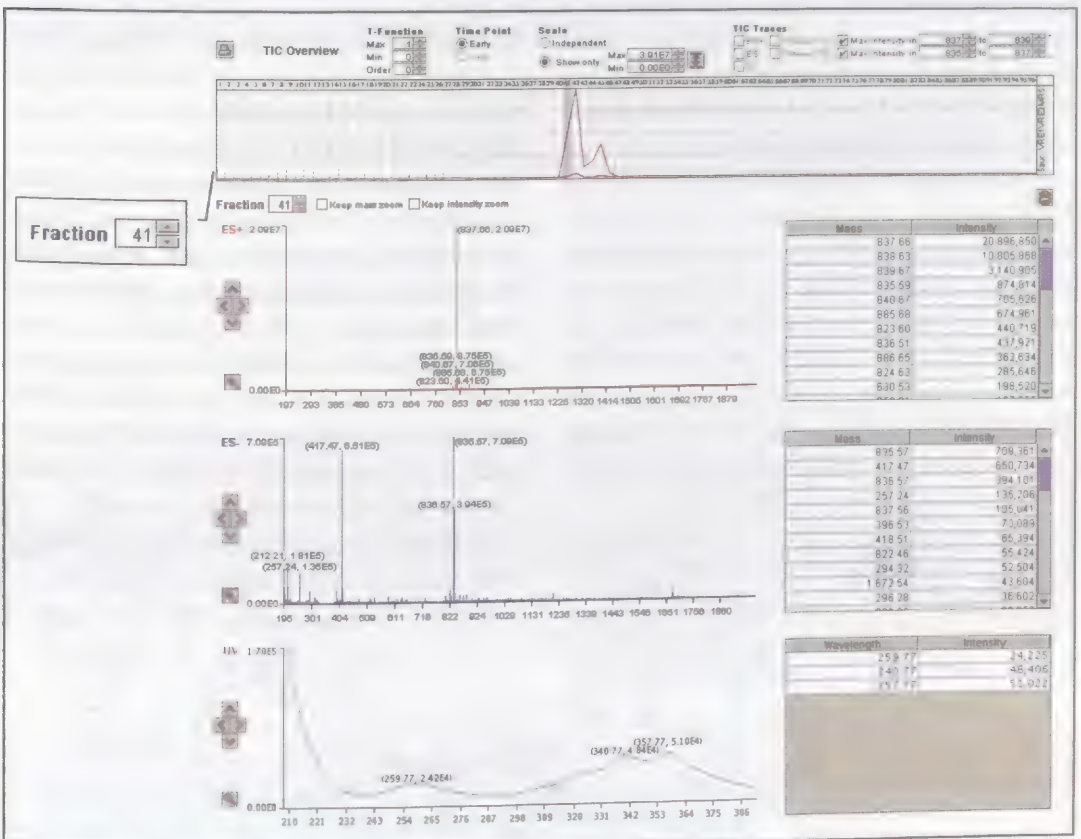

Figure 7. Depiction of one of the IT tools for analyzing the HPLC/UV/MS/bioactivity data from multiple fermentations of *Amycolatopsis orientalis* ATCC 43491.

				235	236	239	278	299	301	322	330	
	AD_1	I M1	I Arg	D	V	W	I	I	G	A	V	Arginine
Microcystin I	McyC	I M1	I Arg	D	V	W	I	I	G	A	V	
	AD_2	I M2	I Ser	D	V	W	H	F	S	L	V	Serine
	AD_4	I M4	I Ser	D	V	W	H	I	S	L	V	
	AD_5	I M5	I Ser	D	V	W	H	L	S	L	V	
CDA I	Pep1	I M1	I Ser	D	V	W	H	F	S	L	V	
Enterobactin I	EntF	I M1	I Ser	D	V	W	H	F	S	L	V	
Daptomycin I	Pep2	I M6	I Ser	D	V	W	H	I	S	L	I	
Syringopeptin I	SypC	I M6	I Ser	D	V	W	H	L	S	L	I	
Syringomycin I	SyrE	I M2	I Ser	D	V	W	H	L	S	L	I	
	AD_3	I M3	I hOrn	D	M	E	N	L	G	L	I	5-N-Hydoxy-ornithine
Coelichelin I	CchH	I M3	I hOrn	D	M	E	N	L	G	L	I	
Exochelin I	FxbC	I M3	I hOrn	D	M	E	N	L	G	L	I	
	AD_6	I M6	I Gln	D	A	Q	E	G	G	L	V	Glutamine
Lichenysin I	LicA	I M1	I Gln	D	A	Q	D	L	G	V	V	
Iturin I	ItuB	I M3	I Gln	D	A	Q	D	L	G	V	V	

Figure 8. Alignment of diagnostic amino acids in adenylylation domains of various NRPSs.

non-toxic, with no observable adverse effect level above 50 mg/kg (both iv and ip), and the drug has shown efficacy in *S. aureus*–induced mouse peritonitis models (Banskota et al., 2006a).

A non-ribosomal peptide synthase (NRPS)–containing locus, also detected in the vancomycin producer, is depicted on this screen. NRPSs are also modular, and the encoded amino acids are determined by amino acids at positions 235, 236, 239, 278, 299, 301, 322, and 330 of the adenylylating enzymes (Stachelhaus, Mootz, and Marahiel, 1999; Challis, Ravel, and Townsend, 2000; Schwarzer, Finking, and Marahiel, 2003). In Figure 8, comparisons are presented for each of the six adenylylating enzymes (AD_1 through AD_6) of this NRPS, with comparable ones for other NRPSs and the secondary metabolites that are produced. The second and last modules of our NRPS include epimerases, and the third module, encoding ω-hydroxyornithine, includes an N-methyl transferase. ORF-3 of the locus (outside the NRPS) encodes an N-formyl transferase, but we were unable to predict the position of formylation from the in silico analysis. This had to

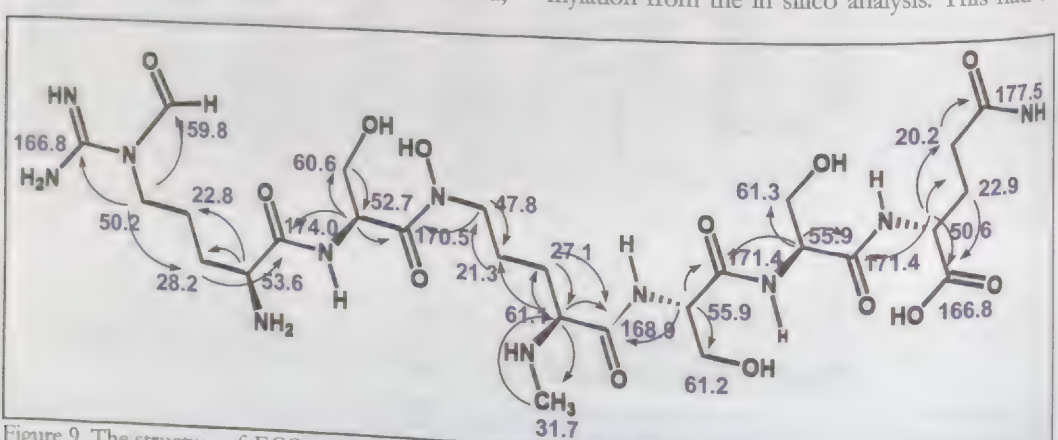

Figure 9. The structure of ECO-0506 with CMR assignments and HMBC (H→C) correlations.

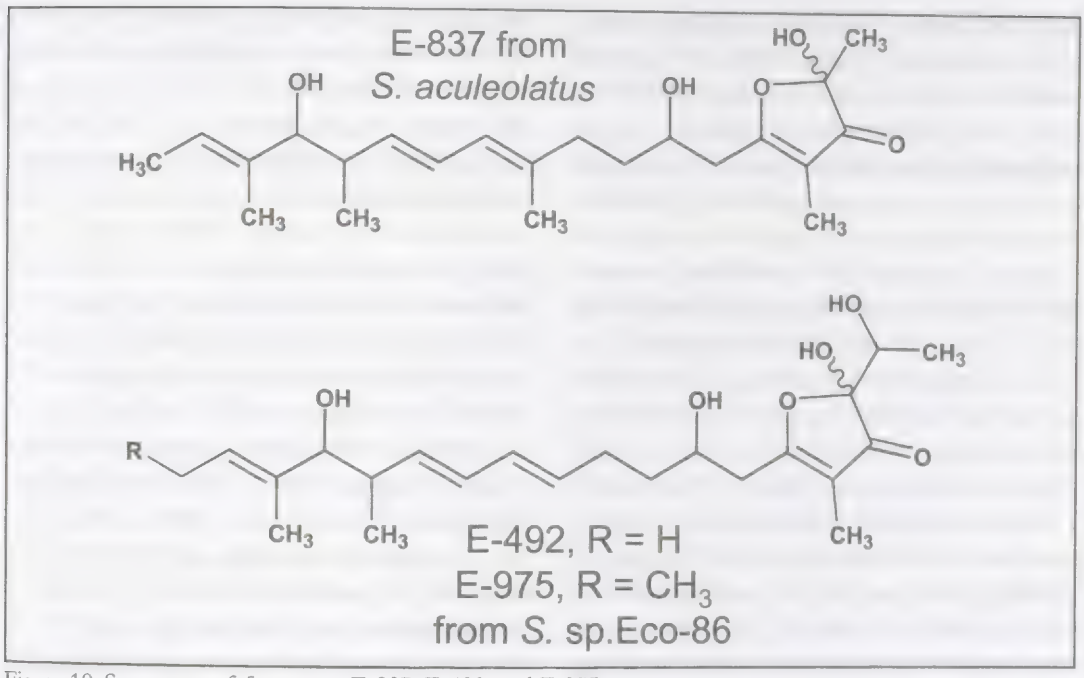

Figure 10. Structures of furanones E-837, E-492, and E-975.

rely on classical 2D NMR spectral analyses of the isolated metabolite. Figure 9 shows the structure of ECO-0506 with the HMBC correlations that place this formyl group on the secondary amino nitrogen of the arginine at position 1. ECO-0506 has only weak antibacterial activity and weak inhibitory activity against ACE and MEK. At 10 μM it potentiates the binding of adenosine to the A3 receptor by 100%.

Can we determine completely new structure types from in silico analysis of the genes? Usually not, because all analyses are based on comparisons with known structures or substructures. It is possible to construct such a structure from known substructures, and this was the case where we found two very similar loci in two different streptomycetes, *Streptomyces aculeolatus* and *Streptomyces* sp. Eco86. Each locus contained a PKS-I encoding a thioesterase, which would be expected to terminate the chain in a δ-lactone. The loci also included similar hydroxylases, and each has an unusual Baeyer-Villiger–type flavin mono-oxygenase. The locus from *Streptomyces* sp. Eco86 also encoded a further hydoxylase for which there was no counterpart in the other strain. There were also differences in some of the ATs of the PKS-I. The fifth and the last ATs of the *S. aculeolatus* PKS-I each specified methylmalonyl-CoA, whereas the corresponding ATs in the sp. Eco86 strain specified malonyl-CoA and ethylmalonyl-CoA, respectively. The strains were grown, the compounds were isolated, and their structures were confirmed as shown in Figure 10 and in accord with the in silico predictions (Banskota et al., 2005, 2006b). E-837 was also isolated from a marine-derived streptomycete (Cho et al., 2006). Neither group has detected any significant biological activity from these structures; however, it is inconceivable that three different strains of actinomycetes would maintain essentially the same 70+ kb locus intact through evolution without a biological activity for the end product. Whether that biological activity will have an application in human therapy is another question.

Another way by which we have used the DECIPHER technology to discover completely

novel structures is from an incomplete structural analysis. This comes about in a situation in which we have a series of genes in the locus, which define partial structures, sufficient to presume novelty, but complemented with a series of genes that have no counterpart in the database. In such a case, one is presented with a high probability of novelty and the opportunity to expand the database into new territory. ECO-4601, Figure 11, currently in the clinic for the treatment of solid tumors, fell into this category. The in silico analysis indicated the presence of a mevalonate-derived poly-isoprenoid chain (probably a straight chain, as there was no evidence of a cyclase) and a 3-hydroxyanthranilic acid moiety. This pair of partial structures, coupled with the fact that the producing strain was a *Micromonospora*, provided a high probability of novelty. After isolation and structure determination, the biosynthetic gene cluster could be analyzed retrospectively and the data added to the DECIPHER database.

Discussion: 2. What Have We Missed?

Honoring the lifetime achievements of Gordon Cragg, and particularly his many productive years leading the Natural Products Branch at the National Cancer Institute (NCI), we should look at natural products from a broad perspective, but as we have been considering microbes, let us start our examination on a microbial scale and look initially at microbes. Again, as we focus, we have been looking at actinomycetes, and although they are perhaps the most studied of the prokaryotes, we find that whole new genera are being discovered in plentiful numbers. Bill Fenical's group alone has added several new genera in recent years as a result of their exploration of the marine environment (Fenical and Jensen, 2006). It is common for the antibiotic discovery programs to be referred to as "soil screening," because most of the microbes entering these projects were isolated from soil samples. Indeed, soil samples have provided a prolific source of a wide variety of different actinomycetes, fungi, and eubacteria. But even here, an analysis of the DNA content of the soil samples suggests that only about 5% of the species are cultivated under generally accepted culture conditions, 95% are "unculturable." Unfortunately, these common colloquialisms create a false impression. Actinomycetes are not confined to soil; they abound in all environments including, for example, cattle rumen (An, Cai, and Dong, 2006). The term "unculturable" almost certainly is defined by a less than exhaustive application of currently used techniques.

The number of "culturable" actinomycete species has been variously estimated between

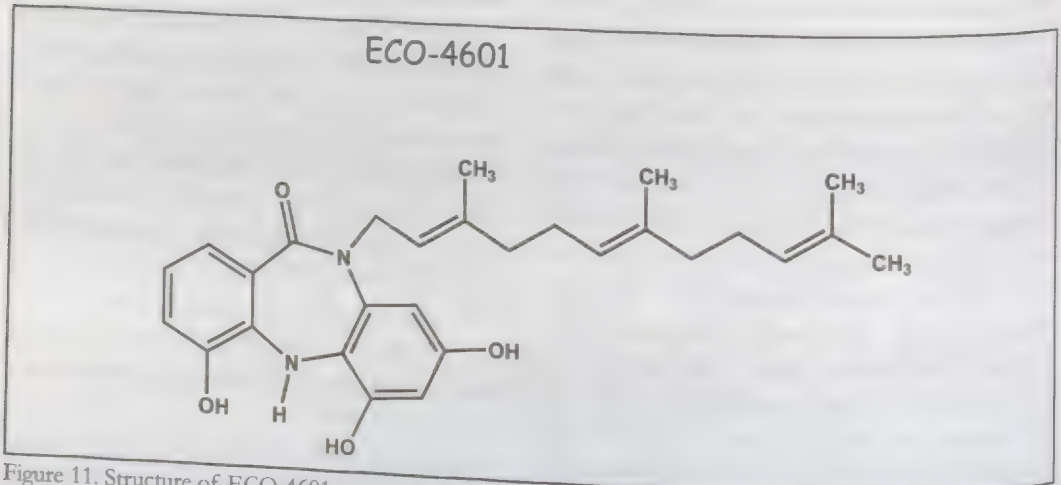

Figure 11. Structure of ECO-4601, currently in phase I clinical trials for the treatment of solid tumors.

35,000 and 50,000, but species have been defined on the basis of morphological characteristics, nutritional growth requirements, and cell wall composition, or, more recently, by 16S-rRNA comparisons. This classification has little to do with secondary metabolic biosynthetic capability. A search of the current edition of Antibase (Laatsch, 2005) for *Streptomyces hygroscopicus* pulls up 291 entries, for which 227 different structures are assigned. These fall into 33 distinct chemical classes. Clearly, there are a large number of different strains of this species, each with its own biosynthetic potential. Rapamycin, for example, was only found in one specific *S. hygroscopicus* strain isolated from a soil sample collected on Easter Island (Sehgal, Baker, and Vézina, 1975).

Moreover, what we find tends to be limited by our screening criteria. The vast majority of microbial-based searches have been directed at the discovery of antibacterials or antifungal compounds. Despite this concentration of effort, the Merck group was able to report (Wang et al., 2006) the discovery, from *Streptomyces platensis,* of platensimycin, a completely novel pharmacophore with potent antibacterial activity. Furthermore, they reported that this metabolite has been found in several strains isolated from a broad geographic distribution.

Natural product discovery has been concentrated in three large source areas: plants, microbes, and marine macro-organisms. Elsewhere in this volume, John Daly describes some of his excellent studies of frog-skin alkaloids, but John's voice was alone in the wilderness while he pursued his study of amphibians. Given the tremendous diversity of species, it is logical to predict that the *Insecta* cry out for systematic investigation of their secondary metabolites. Here we have examined one of the foci of concentration, microbes, where our genomic analyses indicate that even for well-studied organisms, less than 10% of the biosynthetic capabilities have been discovered. Given the small fraction of microbes that have been cultivated and investigated, and the relatively focused nature of the screening, it is clear that we know of less than 1% of the universe of microbial secondary metabolites.

The simplistic idea that antibiotics are produced by microbes to protect them from other microbes has been discredited, and, as Julian Davies points out, antibiotics at concentrations between 1% and 10% of their minimum inhibition concentration induce a large change in transcription in a test organism (Davies, 2006). The exact role played by most antibiotics in the life cycle of the producing organism is, in most cases, a mystery. It defies credibility to assume that a rapidly dividing and mutating organism can maintain tens of thousands of bases aligned to produce distinct complex secondary metabolites without a biological purpose for those molecules. It remains the scientist's challenge to discover these complex compounds.

When it comes to natural product discovery, we have to admit that, to date, we have just scratched the surface. The ability to isolate, purify, and structurally determine minute quantities of compound, and the ability to undertake proteinomics to discern the biological targets of secondary metabolites, will carry this field to a new quantum level.

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Pivotal Connections: Tracing Support by the Natural Products Branch to Drug Discovery from Marine Organisms

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T. Luke Simmons,[1,2] and William H. Gerwick[1,2*]

The Natural Products Branch of the National Cancer Institute, under the leadership of Dr. Gordon Cragg (2006 recipient of the William L. Brown Award for Excellence in Genetic Resource Conservation), has played a key role in facilitating international natural products research as well as multinational training. This manuscript details the participation of the authors' laboratories in several international collaborations, facilitated by the Natural Products Branch, that have led to superb training opportunities for foreign students in the U.S. as well as U.S. students abroad. International sites covered in this manuscript include Curaçao, Madagascar, and Panama. The subjects of our natural products investigations have been a mixture of field-collected and cultured marine cyanobacteria (blue-green algae). These life forms are extraordinarily rich in bioactive natural products derived from mixed non-ribosomal peptide synthetases and polyketide synthases, and several examples illustrating metabolites of this type are described. Additionally, we summarize our precursor feeding and molecular genetic efforts to understand the biosynthesis of some of these structurally intriguing metabolites.

Introduction

The natural products community is highly international, in terms of both its composition (with researchers in the U.S. and elsewhere) and the points of origin of the organisms that are most actively sampled for new and bioactive molecular entities. The Natural Products Branch of the National Cancer Institute (NCI) has long realized that these human and natural resources are international in scope and has taken a leadership role in promoting appropriate and responsible interactions in the world arena. This has included the drafting of an exceptionally well-written, principled, virtuous, and often imitated Memorandum of Understanding for enabling and safeguarding the international collection of biological specimens (National Cancer Institute, 2007). Similarly, the NCI and Natural Products Branch have been a crossroads for international scholars receiving training in the United States. The principal proponent for much of this leadership has been the Chief (1989–2004) of the Natural Products Branch, Dr. Gordon Cragg. For his lifetime efforts to advance our knowledge of natural products, Dr. Cragg was honored in 2006 with the William L. Brown Award, given every two years at the Missouri Botanical Garden in recognition of the outstanding contributions of an individual in the field of genetic resource conservation and use.

Results and Discussion

Curaçao

The initiation of the National Cooperative Natural Products Drug Discovery Group (NCNPDDG) Program in 1989, later re-titled to simply the NCDDG Program, led to the formation of a series of teams with effective partnerships with industry and the NCI (National Cancer Insti-

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tute, 2004). These NCDDGs have proven to be highly effective in discovering new potential anticancer natural products, as well as establishing the connections and infrastructure for the effective pursuit of these leads. The Natural Products Branch of the NCI had a strong influence on the development of this effective NCDDG academic-industrial-government partnership. Indeed, a number of significant compounds have emerged from the NCDDG program, including the bengamides (Crews et al., 2003), psammaplins (Crews et al., 2003), betulinic acid (Balunas and Kinghorn, 2005), pervilleine A (Balunas and Kinghorn, 2005), makaluvamine (Ireland et al., 2003), and neoamphimedine (Ireland et al., 2003). The NCDDGs have been enabled through a significant commitment of resources and time from the NCI, and this has allowed exploration for new anticancer leads with a greater level of sophistication and long-term commitment to the international relationships necessary for significant collections in foreign countries. For example, the NCDDG program enabled our exploration of marine cyanobacteria from Curaçao, which yielded such important compounds as curacin A, barbamide, antillatoxin, and kalkitoxin (Burja et al., 2001; Gerwick, Tan, and Sitachitta, 2001). As detailed below, projects initiated under NCDDG support have expanded in many other dimensions, including molecular pharmacology, chemical synthesis, and biosynthesis. These later experiments include the cloning and investigation of gene clusters involved in natural product biosynthesis.

In 1991 we made initial collections from Curaçao with close interaction with scientists from the Caribbean Research and Management of Biodiversity (CARMABI) research station. This marine laboratory, while modestly funded, has played a pivotal role in characterizing, managing, and safeguarding the marine resources of Curaçao, as well as serving as a conduit for foreign scientists to work effectively within the country. Shallow water collections of a red-colored strain of the cyanobacterium *Lyngbya majuscula* from near the CARMABI station, as well as from mangrove roots in a nearby bay (currently threatened by a housing development!), gave extracts rich in natural products that showed potent activity in several biological assays (Pors and Nagelkerken, 1998). These assays were used to direct the isolation of five distinct classes of natural products, as described below.

The crude extracts showed a variety of activities including cancer cell toxicity, antiproliferative activities, ichthyotoxicity, brine shrimp toxicity, and molluscicidal properties (Figure 1). The cancer cell and brine shrimp toxicity assays led to the isolation of curacin A, B, and C, a novel set of thiazoline and cyclopropyl ring containing lipids of exceptional potency (low nanomolar) (Gerwick et al., 1994). Their structures were first proposed as planar compounds, and subsequent integrated synthetic and degradative sequences were used to establish the configurations at all four stereogenic centers (Nagle et al., 1995). In vitro, curacin A exhibited cytotoxic and antiproliferative activities via binding to the colchicine binding site on microtubules. However, in vivo antitumor effects were not observed due to compound instability (Verdier-Pinard et al., 1998). The combination of curacin A's unique structure, potent anticancer potential, but lack of stability has stimulated numerous chemical synthesis efforts, including elegant combinatorial chemistry efforts by the Wipf group aimed at producing a stabilized analog of curacin A (Wipf et al., 2002). In our laboratory and that of our collaborator, David Sherman at the University of Michigan, we have investigated the biosynthesis of curacin A through a combination of precursor feeding experiments and characterization of the biosynthetic gene cluster (Chang et al., 2004). These studies have shown that the majority of the molecule is produced from acetate (Figure 2A). In addition, two methyl groups derive from S-adenosylmethionine (SAM), and the entire atom composition of cysteine is used in forming the thiazoline ring. Perhaps most intriguing, the methylene group of the cyclopropyl ring

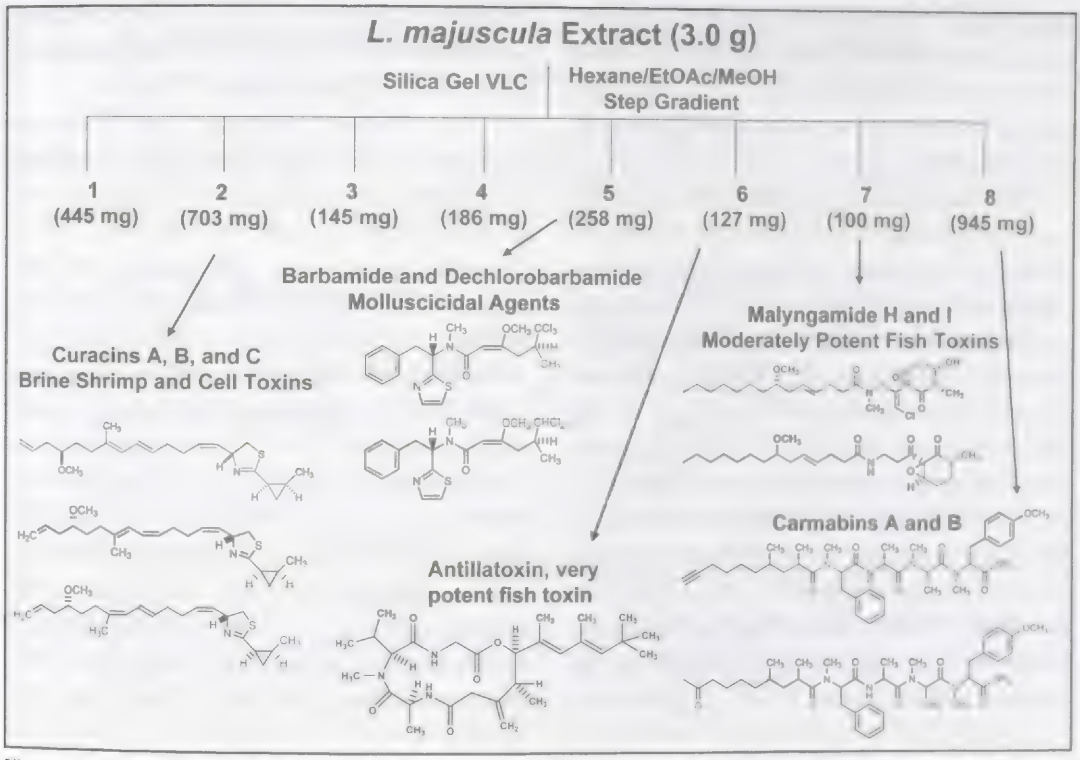

Figure 1. Fractionation chart showing the isolation of various classes of bioactive metabolites from a Curaçao strain of *Lyngbya majuscula*.

was shown to derive from C-2 of acetate, indicating a branched polyketide origin. Cloning and sequencing of the gene cluster gave new insight into the metabolic origin of this group, as an 8 kb gene cassette was identified containing a 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) synthase-like domain at its core, which codes for proteins that are likely responsible for creation of this 3-mem-

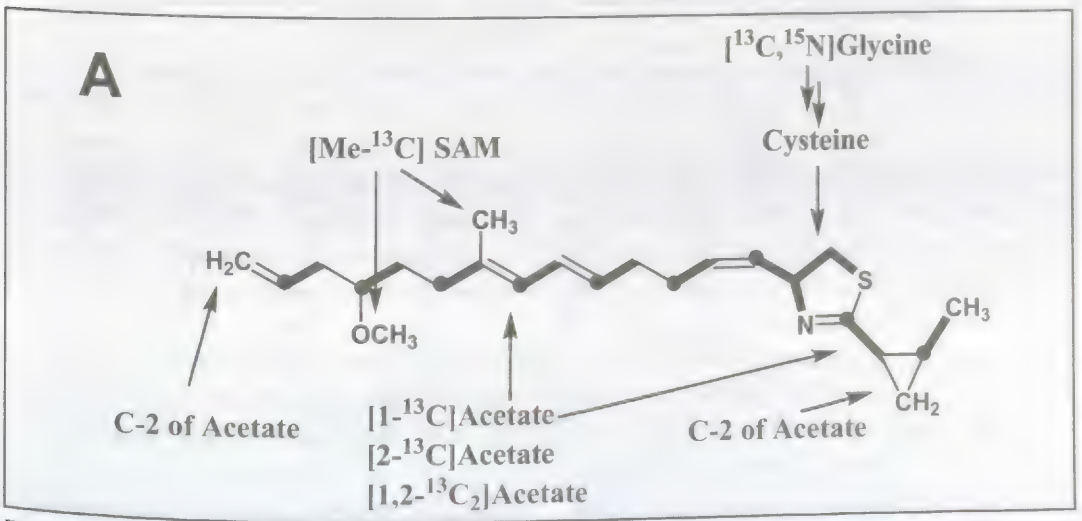

Figure 2A. The biosynthesis of curacin A from isotope-labeled feeding experiments.

bered ring (Chang et al., 2004). Recently, our combined laboratories have expressed proteins from this cassette that clarify some of the steps that lead to cyclopropyl ring formation (Figure 2B) (Gu et al., 2006).

Further analysis of biological extracts in a fish toxicity assay resulted in the isolation of two new classes of compounds, including the cyclic lipopeptide antillatoxin (Orjala et al., 1995) and the linear lipopeptide malyngamide H (Orjala, Nagle, and Gerwick, 1995) (Figure 1). Antillatoxin exhibited extremely potent ichthyotoxicity (LD_{50} = 50 ng/ml.) and has been the subject of chemical synthesis as well as more detailed pharmacological studies. For example, the two stereocenters associated with the lipid section were found to be critical for its potent biological activity (Li et al., 2004). Further studies revealed that antillatoxin displays potent neurotoxicity due to its ability to activate voltage-gated sodium channels (VGSC) in mammalian cells (Li et al., 2001). Its site of action was inferred (through co-administration with other VGSC-binding ligands, such as batrachotoxin and brevetoxin) to be at a novel location on the VGSC.

The molluscicidal properties of the extract were localized to a modified peptide natural product present as several percent of the lipid extractables. Spectroscopic properties were used to determine its structure—barbamide, a modified tripeptide containing a thiazole ring and, most uniquely, a trichloromethyl group (Orjala and Gerwick, 1996). Subsequent isotope-labeled precursor feeding experiments delineated the source of essentially all atoms in the molecule, including leucine, phenylalanine, a decarboxylated cysteine, and N- and O-methyl groups deriving from SAM (Sitachitta et al., 2000). In a fashion parallel to curacin A, the biosynthetic gene cluster was cloned, sequenced, and found to be essentially co-linear with a sequence of reactions necessary to create barbamide (Chang et al., 2002). Work in our laboratory (Flatt et al., 2006) and that of Chris Walsh at

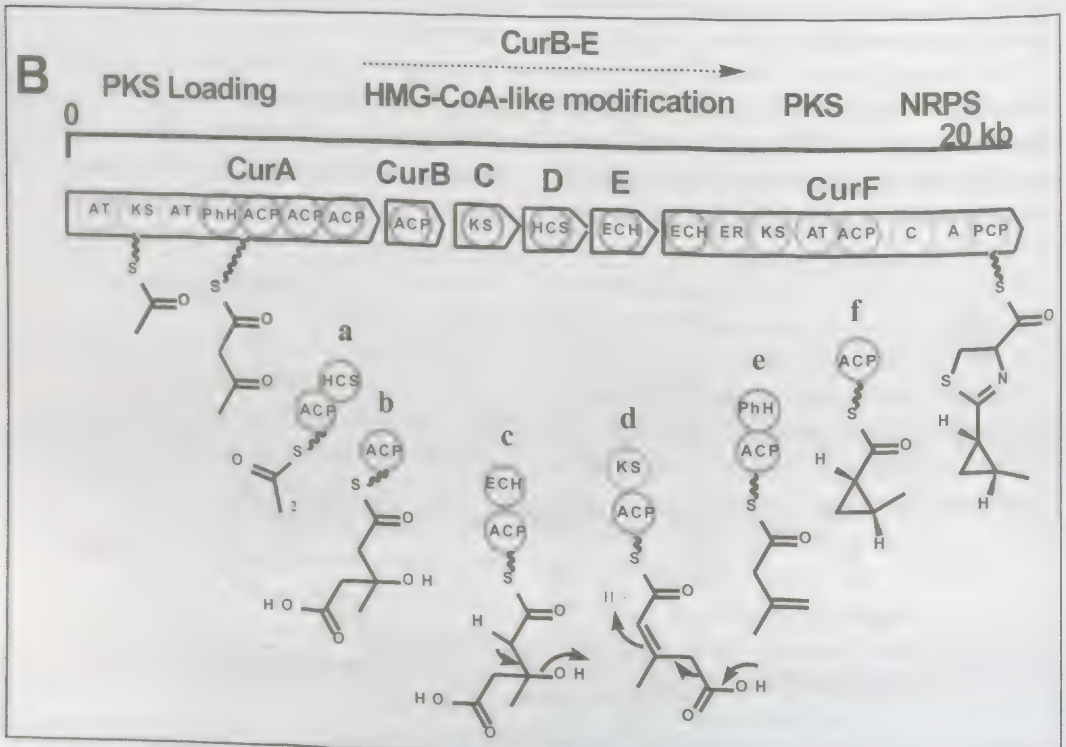

Figure 2B. Details of cyclopropyl ring formation by proteins curA to curF of the curacin A biosynthetic gene cluster.

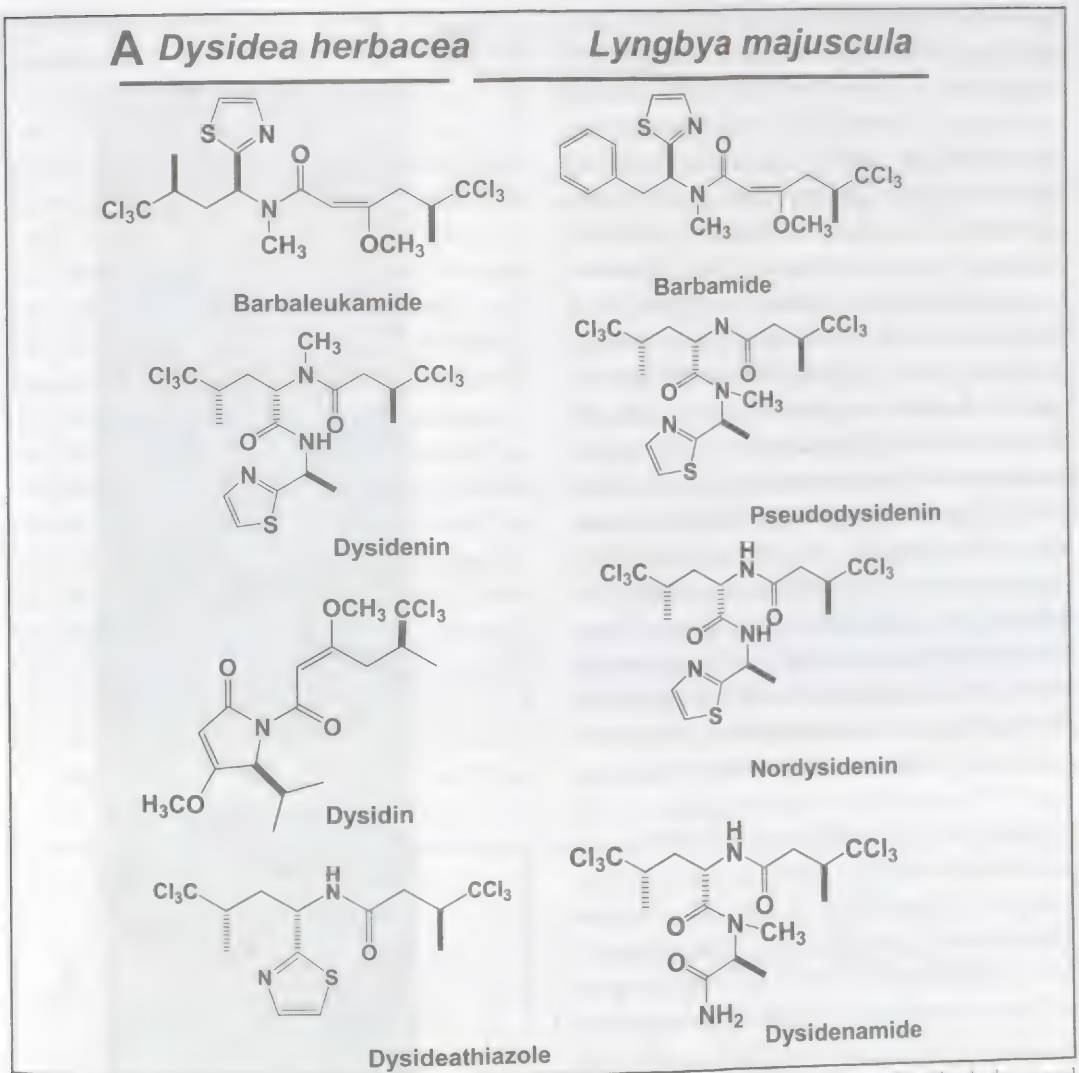

Figure 3A. Comparison of the chlorinated peptide metabolites isolated from the sponge *Dysidea herbacea* and the cyanobacterium *Lyngbya majuscula*.

Harvard (Galonic, Vaillancourt, and Walsh, 2006) has identified the BarB1 and BarB2 proteins as the catalysts for the sequential dichlorination of the pro-*S* methyl group of leucine; they then convert this to the trichloromethyl group observed in barbamide. Mechanistically, we first realized from deuteroleucine feeding experiments that chlorination must be occurring by a novel mechanism, likely involving radicals (Sitachitta et al., 1998). This hypothesis received partial confirmation when we cloned the gene cluster and found good homology between the BarB1/BarB2 proteins and phytanoyl-CoA hydroxylase (Chang et al., 2002). Detailed and elegant biochemical experiments in the Walsh laboratory have confirmed this hypothesis and given deep insight into the mechanism by which this transformation occurs (Galonic, Vaillancourt, and Walsh, 2006).

Early in the barbamide structure project we recognized the striking structural similarity to a number of *Dysidea* "sponge" chlorinated peptides, such as shown in Figure 3A. Indeed, others have

also noted this parallel, and together it was proposed that the cyanobacterial symbiont (*Oscillatoria spongeliae*) found intermixed with the sponge cells was actually responsible for chlorinated peptide production. Cell sorting experiments supported this hypothesis, as these peptides were found to be associated with purified cyanobacteria populations (Faulkner, Unson, and Bewley, 1994). However, because the cyanobacterium was not able to be cultured, demonstration of de novo biosynthesis was precluded, and it is conceivable that the peptides were produced by another cell type, excreted, and then absorbed into the wall matrix of *O. spongeliae*. Isolation of the barbamide halogenases BarB1 and BarB2 allowed a genetic-based approach to investigate and confirm the identity of the producing organism in this symbiosis (Flatt et al., 2005). First, homology-based cloning from the sponge/cyanobacterial mix yielded a *barB1* homolog, *dysB1*, encoding a protein sequence with 93% identity to BarB1. The *dysB1* sequence, in combination with cyanobacterial-specific 16S rDNA sequences, was used in fluorescence in situ hybridization (FISH) experiments to probe thin sections of the sponge containing the cyanobacterial symbiont. Cyanobacteria were identified in these thin sections by their chlorophyll fluorescence and by localization of a blue fluorescent-labeled probe specifically designed to bind to cyanobacterial 16S RNA sequences. The final experiment localized the fluorescently labeled *dysB1* probe relative to the location of sponge and cyanobacterial cells (Figure 3B). The probe bound exclusively to the cyanobacterial cells, thus showing unequivocally that the cyanobacteria possess the halogenase necessary for chlorinated peptide biosynthesis. This was the first example of a genetic-based approach to determine the site of biosynthesis of a natural product obtained from a complex marine symbiosis (Hildebrand et al., 2004).

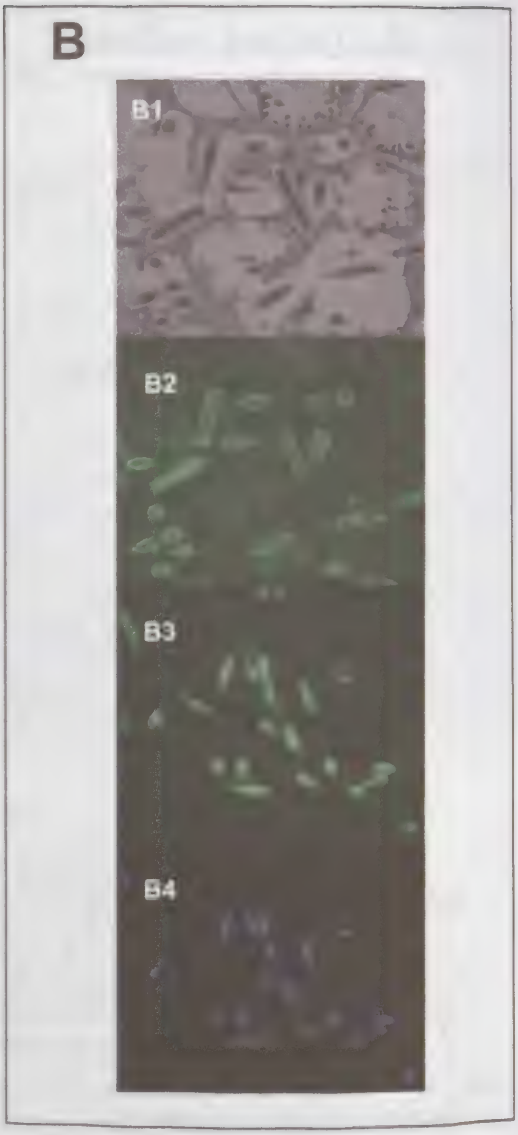

Figure 3B. CARD-FISH analysis, using a fluorescently labeled halogenase probe and thin sections of *Dysidea herbacea*/*Oscillatoria spongeliae*, shows that the site of biosynthesis of chlorinated peptides is within cyanobacterial cells. (B1 = thin section of O-tolidine–stained sponge/cyanobacterial tissue under normal light; B2 = the same thin section viewed by autofluorescence emission at 552 nm; B3 = a second thin section of sponge/cyanobacterial tissue showing location of cyanobacterial cells by autofluorescence at 552; B4 = the same section as B3 but showing cellular binding of an Alexa-Fluor-labeled complimentary oligonucleotide to the halogenase gene sequence *dysB1* cloned from the sponge/cyanobacterial tissue.)

Madagascar

The Natural Products Branch of the NCI has played a crucial role in developing international

relationships among scientists of many countries, especially those with developing scientific infrastructure. Through continued support from the NCDDG program, we were able to make connections, via South African scientist Professor Mike Davies-Coleman of Rhodes University, that led to collection opportunities in Madagascar during the late 1990s. Additional collection and education trips have ensued, including one in 2004 to assist the Kingston International Cooperative Biodiversity Group (ICBG) program in their initiation of a marine collection program. Additionally, the connection with Professor Davies-Coleman has provided a conduit to several superbly trained and enthusiastic postdoctoral scientists, namely Drs. Greg Hooper, Kerry McPhail, Edith Antunes, and Denzil Beukes. Collectively, these scientists have contributed enormously to the productivity of our cancer-drug discovery program over the past decade. One example is given below.

A sizable number of gelatinous red mass of cyanobacteria were collected in 15–20 m of water depth off Nosy Be on the northwest coast of Madagascar. The taxonomy of this material was difficult to establish; however, eventually, a close morphological match was obtained with the genus *Geitlerinema*. Subsequently, this collected material was extracted, fractionated over silica gel, and evaluated for toxicity against brine shrimp. Interestingly, most of the fractions showed quite potent effects in this model, and follow-up isolation efforts have yielded three distinct classes of toxic molecules.

The first to be examined was a lipophilic fraction. Quite quickly it was realized that this fraction contained curacin A. That this cyanobacterium from the South Indian Ocean contained the same compound as originally obtained from a Caribbean collection of *Lyngbya majuscula* is quite remarkable and suggests a wide taxonomic and geographic distribution of this gene cluster. The second series of compounds were closely related macrolides of complex structure, highly toxic to brine shrimp (Figure 4A). Fortunately, large portions of their structures were closely related to a

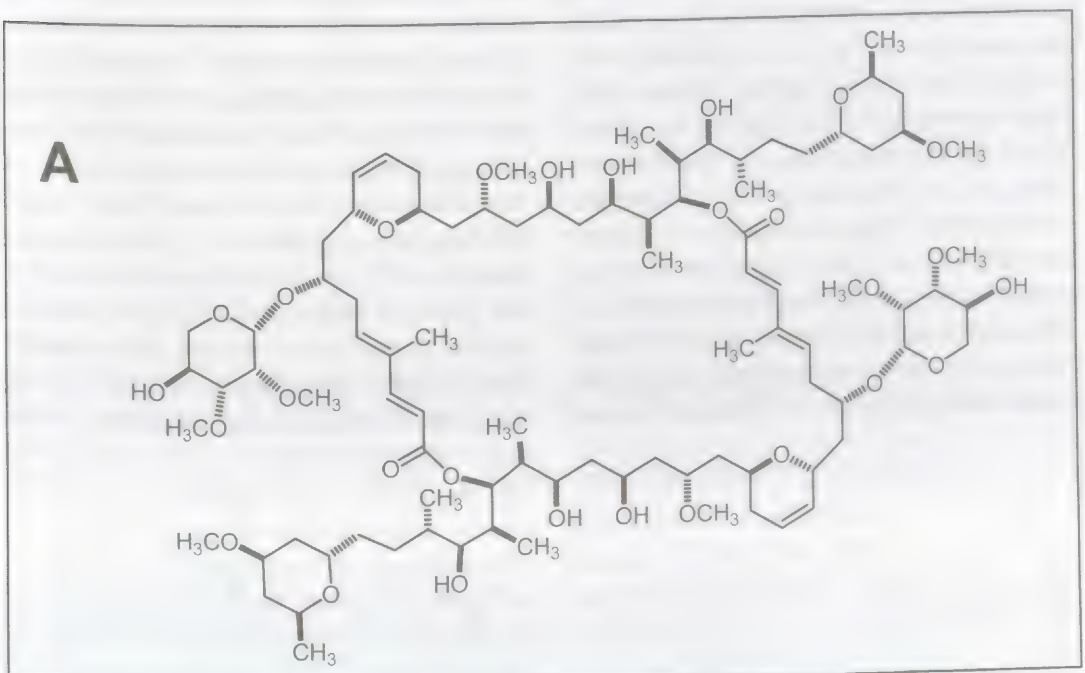

Figure 4A. Structure of metabolite ankarharolide A obtained from *Geitlerinema* sp. collected subtidally from northwestern Madagascar.

well described series of "sponge" compounds, the swinholides (Kobayashi et al., 1989), greatly simplifying the elucidation of their structure. In fact, the major compound, ankarharolide A, was nearly identical to swinholide A, with the addition of a dimethoxy sugar to each half of the dimer that forms the swinholide dilactone structure (Andrianasolo et al., 2005). Ankarharolide A was characterized using 2D nuclear magnetic resonance (NMR) techniques, and defined as the first glycosylated swinholide. This new compound was shown to be highly toxic to cells through interference with actin polymerization, and in this regard, has a similar mechanism of action to the swinholides. Perhaps most significantly, the isolation of this family of compounds from a marine cyanobacterium gives insight into the metabolic origin of the swinholides in sponges. Similar to the barbamide-like compounds found in *Dysidea herbacea*, swinholide production is predicted to occur within cyanobacterial symbionts found in association with the sponge *Theonella swinhoei*.

In addition to the ankarharolides, a slightly less polar fraction was also toxic to brine shrimp and showed the presence of a predominant metabolite. Reverse-phase high-performance liquid chromatography (HPLC) was effective in the purification of this new compound, named "mitsoamide" (Figure 4B) (Andrianasolo, Goeger, and Gerwick, 2007). High-resolution mass spectrometry (MS) (m/z 1093.6835) gave a molecular formula of $C_{55}H_{96}ClN_9O_{11}$, suggesting a metabolite with a substantial peptide component. ^{1}H NMR features were not tremendously revealing as the signals were broadened, and there was evidence of N-methyl amide rotomers leading to multiple conformers in solution. The ^{13}C NMR, on the other hand, clearly showed a series of amide-type carbonyls typical for peptide-containing metabolites. Structure elucidation of this relatively large and complex metabolite was possible using a series of 2D as well as very effective 1D NMR experiments, such as the 1D heteronuclear multiple bond correlation (HMBC) and 1D total correlation spectroscopy (TOCSY). The 1D and 2D NMR experiments first defined the spin systems of five standard amino acid residues, namely valine, leucine, alanine, phenylalanine, and a C-terminal residue of N,O-dimethylisoleucine.

Continued analysis of these NMR data sets allowed formulation of three additional residues of an unusual character. The first was a cyclohexyl aminal for which HMBC provided a highly overlapped network of correlations. This was strongly assisted by a 1D TOCSY, which revealed all of the proton resonances of this cyclic system except those on the basic nitrogen. The branching nitrogen was present as an amide, and hence its proton was readily observed and contributed to the spin system information. Ultimately by process of elimination and supported by chemical shift models and mass spectrometry, the basic nitrogen in the ring was identified as possessing two protons and thus carrying positive charge; the counter ion was shown to be chlorine by mass spectrometry. A second unusual partial structure was shown to be a homolog of lysine, possessing one more carbon in the side chain. Interestingly, the alpha amino group was free while the side chain ζ nitrogen was again present in an am-

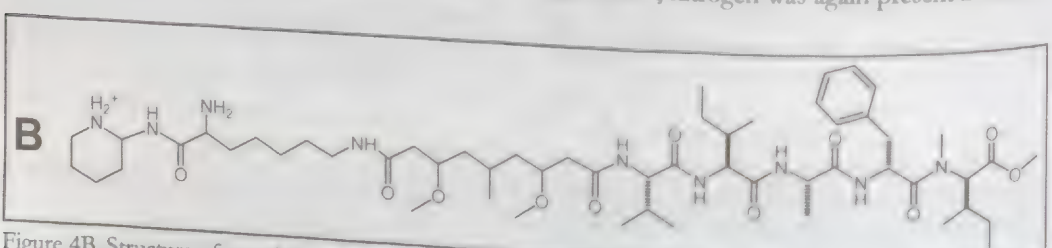

Figure 4B. Structure of metabolite mitsoamide obtained from *Geitlerinema* sp. collected subtidally from northwestern Madagascar.

ide bond with another residue. A combination of NMR data was used to support this partial structure, with particular insights being gained from the HMBC and 1D TOCSY experiments. The final partial structure was a symmetrical nonadioic acid with two methoxy and one methyl substituent. The same set of NMR experiments was used to define this structure, and, notably, irradiation of the mid-field methylenes adjacent to the amide carbonyls at δ2.23–2.43 gave consecutive TOCSY transfer to the alpha methoxy protons at δ3.59, a high-field methylene at δ1.39–1.49, presumably an adjacent methane at δ1.39, and remarkably, a high-field methyl group at δ0.91, representing four consecutive TOCSY transfers.

Additional HMBC correlations allowed assembly of these eight partial structures into a linear lipopeptide with one terminus given by the N,O-dimethyl isoleucine residue and the other by the cyclic aminal. This structural hypothesis was given additional confirmation by a combination of electrospray ionization (ESI) MS/MS and fast atom bombardment (FAB) MS, which gave in composite a rich number of fragmentations. The configurations of the five standard amino acids were revealed by chiral HPLC of the acid hydrolysate along with standards and showed that all but the phenylalanine residue was of L-configuration (Figure 4B). The remaining five asymmetric centers were not determined due to lack of standards and the relatively low amounts of the isolated compound (3 mg) (Andrianasolo, Goeger, and Gerwick, 2007).

Evaluation of mitsoamide to several cancer cell lines revealed it to be a potent cytotoxin, with an IC_{50} = 460 nM to the NCI's H-460 human lung cancer cell line. Mitsoamide is a new chemotype of lipopeptide from cyanobacteria and possesses a number of novel features: a cyclic aminal, homolysine, and nonadioic acid. However, in a very general way, it fits the essential pharmacophore model for antitubulin lipopeptides; it has a positively charged nitrogen atom at one end, a number of aliphatic groups through the middle portion, and terminates

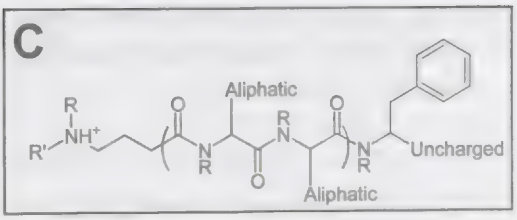

Figure 4C. A pharmacophoric model for highly bioactive linear peptides and lipopeptides, generated, in part, from mitsoamide.

on the C-terminus with an uncharged aromatic residue (Figure 4C). This general model holds true for a number of the dolastatins (Pettit, 1997) and symplostatins (Luesch et al., 2002), belamide (see below), and now mitsoamide.

Panama

At the 2001 American Society of Pharmacognosy annual meeting in Oaxaca, Mexico, connections were made with the Panama ICBG, a program under the administrative leadership of the Fogarty International Center (John E. Fogarty International Center, 2008). The Natural Products Branch, under the guidance of Gordon Cragg, had much to do with the genesis of this inspired program. The Panama ICBG seeks to intimately connect the process of drug discovery from natural sources with the scientific training of U.S. and foreign personnel at many levels, develop infrastructure in biodiversity-rich but economically disadvantaged countries, aid in economic development, promote conservation, and conduct inventories of biodiversity. The aforementioned connections were facilitated in part by the Natural Products Branch and have led to a deep involvement in the Panama ICBG. In the 2003 renewal of the Panama ICBG, currently headed by one of us (Bill Gerwick), a strong host country involvement in all aspects of the science of this endeavor has been maintained, and this continues as a distinctive feature of the Panama ICBG. Overall, the Panama ICBG program has had success on many fronts, including extensive training of Panamanian, as well as U.S.,

students in natural products drug discovery, tropical disease biology, botanical and conservation research, and conservation projects focused on Coiba, an island off the Pacific coast of Panama. In 2003, the former prison island was designated a Panamanian National Park, and preservation of this unique lowland jungle was further secured this past year by its designation as a World Heritage Site (UNESCO World Heritage, 2005).

Collection of marine algae and cyanobacteria has occurred under permit authority of the Panamanian Ministry in charge of conservation, Autoridad Nacional del Ambiente (ANAM), in national park areas under its jurisdiction. Significant surveys of the marine flora from Coiba and the Caribbean locations of Bocas del Toro and Portobello have yielded several hundred collections of algae for screening through tropical diseases (malaria, leishmaniasis, Chagas diseases) and cell-based, as well as mechanism-based, anticancer assays. From these efforts, a number of marine cyanobacteria of the genera *Lyngbya*, *Oscillatoria,* and *Symploca* have yielded a wealth of bioactive natural products; two of these investigations are briefly described below.

A red-pigmented *Lyngbya* sp. from Bastimentos Reef in the Bocas del Toro area was subjected to fractionation using vacuum liquid chromatography, and the fractions were then evaluated in the tropical disease assays listed above. A moderately polar fraction was found to have reasonable activity to a drug-resistant strain of *Plasmodium falciparum* and thus was further processed by HPLC to yield a new compound, which was given the common name of "dragomabin" in recognition of its structural relationship to two other compounds previously in the literature: "carmabin" from a Curaçao strain of *Lyngbya* (Hooper et al., 1998) and "dragonamide" from a Panama strain of *Lyngbya* obtained from Bocas del Drago (Jiménez and Scheuer, 2001; Chen et al., 2005) (Figure 5A). Indeed, careful analysis by TOCSY, correlated spectroscopy (COSY), and heteronuclear single quantum coherence (HSQC) revealed a series of amino acids, including N-methylphenylalanine, one alanine and one N-methylalanine, and an N,O-dimethyltyrosine residue. Additionally, a lipid section containing a terminal acetylene and a single secondary methyl group was defined by these techniques. The only

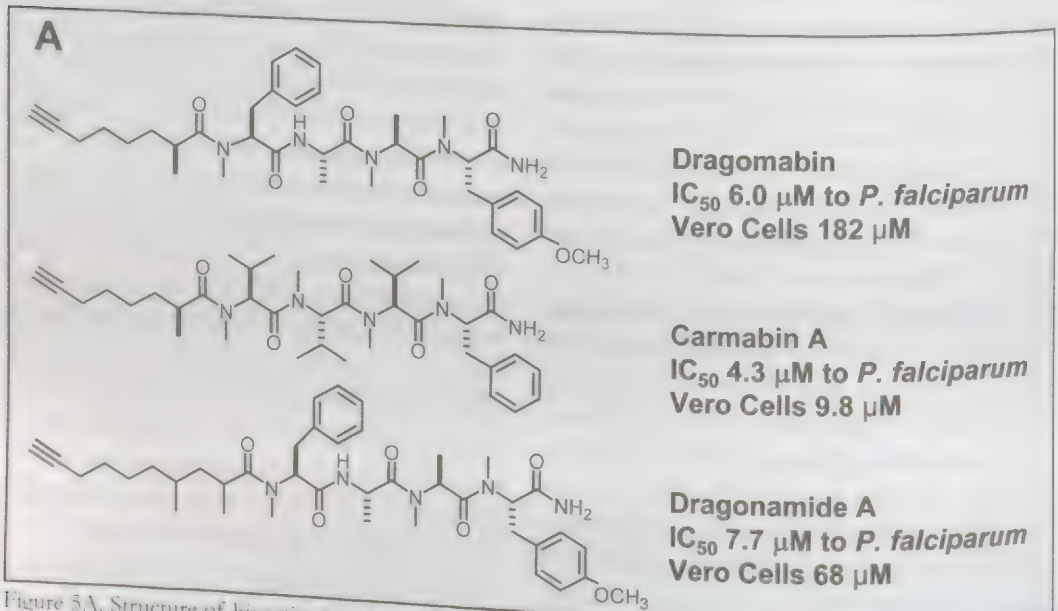

Figure 5A. Structure of bioactive metabolite dragomabin, a new antimalarial lipopeptide, and the related compounds carmabin and dragonamide.

remaining atoms not accounted for by these partial structures were one nitrogen and two hydrogens. By additional HMBC NMR analysis, these atoms were shown be present as a terminal amide group. HMBC, NOSY, and MS fragmentation data were then utilized to efficiently sequence this linear lipopeptide, developing a molecular structure that is a conceptual fusion of carmabin (amino acid portion) and dragonamide (lipid portion) (McPhail et al., 2007). Configurations of the amino acids were established by standard hydrolysis and Marfey's analysis in comparison with authentic standards. Due to the isolation of related compounds from related cyanobacterial isolates collected in relatively close proximity to one another, the *S*-configuration of the stereocenter in the lipid section can be reasonably hypothesized to be the same as that of dragonamide (Jimenez and Scheuer, 2001; Chen et al., 2005). The pure compound was moderately potent in the *H. folkjarnae* assay (IC_{50} = 6.0 μM) and showed a good differential relative to the host Vero cells (IC_{50} = 182 μM) (McPhail et al., 2007).

Another example of a bioactive linear peptide (from a Panamanian marine cyanobacterium) comes from a deeper water collection of *Symploca* obtained from the Portobello area in Panama. Scuba was used to make collections of this orange-colored cyanobacterium in modest quantity, and initial assays showed an exceptional level of cytotoxicity to breast cancer (MCF-7) cell lines ($IC_{50} \leq$ 0.007 μg/mL). However, only a moderate cancer cell toxin, named "belamide A," could be isolated by bioassay-guided fractionation techniques (Figure 5B) (Simmons et al., 2006). It is possible that a more active substance decomposed during isolation or that the original assay data were in error. Nevertheless, belamide A is an interesting compound in its own right and gives some exciting insights into the biosynthetic capacities of marine cyanobacteria as well as structure-activity relationships of linear peptides such as dolastatin 10 and 15 (Flahive and Srirangam, 2005).

It was relatively easy to determine that the planar structure of belamide A contained an

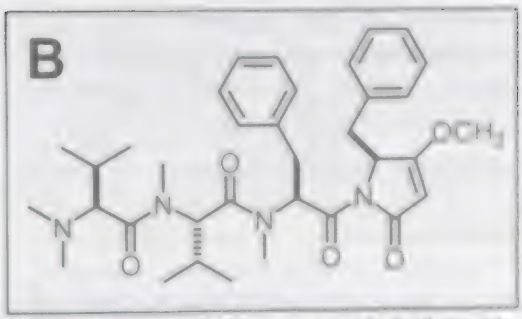

Figure 5B. Structure of bioactive marine lipopeptide belamide A, a new cancer cell antiproliferative agent, from Panamanian collections of marine cyanobacteria.

N,*N*-dimethylvaline termini, an additional *N*-methyl valine, *N*-methyl phenylalanine, and a second, further modified phenylalanine, thereby representing a truncated version of the longer peptides dolastatin 10 and 15. The modification of the phenylalanine residue, deciphered by careful HMBC data analysis, was shown to represent an acetoacetamide Phe which then cyclizes to a pyrrolinone ring. The resulting β-ketone functionality is further modified by enolization and *O*-methylation, the same as present in dolastatin 15. HMBC in concert with tandem ESI mass spectrometry were key to sequence analysis of the peptide, and configurations of all stereocenters were determined by chiral HPLC analysis both before and after ozonolysis (Figure 5B). Biochemical evaluation showed belamide A to be a classic tubulin depolymerizing agent at 20 μM, and it was reasonably active as an antiproliferative agent to the HCT-116 cell line (IC_{50} = 0.74 μM). Hence, the concept that the *N*,*N*-dimethylvaline residue is important to the activity of antimitotic peptides of this sort appears correct, but the decreased number of residues between termini appears to reduce potency rather markedly (Flahive and Srirangam, 2005; Simmons et al., 2006).

Continuing investigations of these and other collections of marine cyanobacteria from the Caribbean coast of Panama have yielded additional linear lipopeptides with powerful biological properties. It is interesting to note that these southern

Caribbean collections are rich in linear (as opposed to cyclic) peptides and lipopeptides; collections from elsewhere in the world (e.g., the South and Indo-Pacific regions) have yielded many more examples of the cyclic varieties.

CONCLUSIONS

Marine cyanobacteria have been a rich source of novel secondary metabolites for nearly three decades, beginning with the pioneering work of Richard Moore at the University of Hawaii and continuing with our work and that of others over the last 15 years (Burja et al., 2001; Gerwick, Tan, and Sitachitta, 2001). Despite this fairly long period of effort, these organisms continue to yield novel secondary metabolites of unusual, sometimes surprising, chemical structure, many of which possess potent biological activities of potential utility in treating human diseases. The results described above give further evidence for the exciting and diverse biosynthetic capacity of marine cyanobacteria and raise interesting questions concerning the molecular evolution of these pathways and the significance to the adaptive strategies of these organisms (Nagle and Paul, 1999). That they are important goes without question; such elaborate compounds require considerable allocation of metabolic energy to produce. Moreover, in the few cases available for analysis, the more advanced forms of cyanobacteria (e.g., *Nostoc punctiforme*, *Anabaena*, and *Lyngbya*) possess a wealth of modular-type non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) pathways, allocating between 5% and 10% of their genetic information to the encoding of secondary metabolite pathways.

Tremendous progress is being made toward understanding the mechanistic chemistry, enzymology, and genetic encoding of natural products biosynthesis. These efforts are revealing that marine cyanobacteria have an abundance of highly refined secondary metabolite biosynthetic pathways, mainly of a modular PKS or NRPS type. The high level of refinement that is evident in the metabolic pathways of cyanobacteria may reflect the long period over which they have evolved, thus justifying the moniker "the world's oldest natural products chemists." Continued study of the biosynthesis of cyanobacterial natural products is revealing fundamentally new mechanistic biochemistry, such as halogenation by radicals, addition of pendant-branching carbons in polyketides via terpene-like reactions, and pathway-terminating reactions that result in small heterocycles or decarboxylated products. Ultimately, we will learn how to harness these unique metabolic capacities and engineer new molecules of utility in biomedicine, and this will usher in a new era of integrated natural products chemistry and molecular biology.

Finally, the natural products sciences represent a superb opportunity for training in the international arena. In part this stems from the fact that some of the world's richest habitats are found in countries with poor capacities in which to perform these studies, but because of their long contact with their unique biota, many individuals have an inherently high enthusiasm and interest. Hence, relatively small investments in terms of in-country laboratory development and training can have a huge impact, helping to initiate a cultural shift towards increasing the value of scientific investigation in the developing world. Bilateral training programs are especially beneficial. Training of young U.S. scientists in the developing world teaches a cultural understanding and sensitivity that is difficult to assimilate in any other way and provides for the maximal input to the host country in terms of contact with a U.S.-trained scientist. Reciprocally, a formal U.S. training program for meritorious students from developing countries allows development of skills and knowledge at much greater depth. Many aspects of the natural products sciences are technically and intellectually accessible at early stages in a student's training, and thus can give very positive feedback and encouragement at a critical time of development. In these regards, the Natural Prod-

ucts Branch of the NCI, under the leadership of Gordon Cragg, has played a pivotal role in the development of many scientists (both in the U.S. and elsewhere), developed relationships favorable to these interchanges, and promoted respect and integrity among participants working in the international arena.

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Legal and Ethical Issues:
From the Convention on Biological Diversity to Conservation

Bioprospecting in the Cragg Era: Accomplishments and the Road Ahead

Michael J. Balick[1,2]

The natural products research community anticipated the basic elements of the Convention on Biological Diversity well before its signing in 1992, recognizing the need to share benefits with both countries and communities based on their contribution of sovereign plant genetic resources and traditional knowledge, both of which are critical elements in drug discovery efforts. The Natural Products Branch of the Developmental Therapeutics Program of the National Cancer Institute (NCI) played an innovative and key role in fostering equitable collaborative relationships in countries where plant material was collected via contracting institutions, in its program to supply raw materials for the discovery of new anticancer, and later, new anti-HIV drugs. These agreements, which came to be known as NCI's Letter of Intent and Letter of Collection, arose from deliberations in the late 1980s and quickly became the standard, not only for NCI's benefit-sharing framework in many countries in which it worked, but also for many academic and commercial agreements as well. In the rich and lengthy dialog that has followed the Convention on Biological Diversity entering into force in 1993, the NCI program serves as a model mechanism for access and benefit sharing policies, including building scientific capacity in developing countries. The New York Botanical Garden received an NCI collections contract in 1986 to work in the Neotropical region. As part of that project, our group implemented a project in Belize to inventory the biodiversity and traditional uses of plants by local people, which over the years grew into a nationwide effort. Gordon Cragg played a vital role in making these NCI collection projects possible in many areas of the world, and his contributions will be discussed.

Introduction

It was a great pleasure to learn from Jim Miller that the William L. Brown Center of the Missouri Botanical Garden would bestow upon Dr. Gordon Cragg its highest honor, the William L. Brown Award, in recognition of his work in natural products research and the search for novel therapies for human illness. This recognition and symposium in his honor are particularly fitting, as Gordon Cragg has aided so many scientists and students working in the natural products field, as well as influenced the thinking of policy makers and others regarding the importance of biodiversity and genetic preservation. My first interactions with Gordon were through his work at the Natural Products Branch of the Developmental Therapeutics Program (DTP) at the National Cancer Institute (NCI), and over several decades I have come to know him as a friend and colleague.

In 1986, The New York Botanical Garden (NYBG) was awarded a collections contract for the Neotropical region and was charged with collecting higher plant samples for study in the NCI's laboratories. At the inception, the project was focused on identifying compounds with anticancer activity via a series of in vitro screens, with anti-HIV screens added several years later. One of our earliest discussions was at a contractor's meeting at the NCI, in which three groups—from NYBG, the Missouri Botanical Garden, and the University of Illinois at Chicago—received guid-

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[2]I am grateful to my many colleagues at NYBG who contributed to the work described herein, in particular Drs. Douglas Daly and Hans Beck, who were co-PIs on the NCI contract. In addition, thanks are due to the hundreds of local individuals and plant taxonomists who made it possible to collect and identify the specimens sent to the NCI for study. Dr. James Miller was kind enough to invite me to the meeting in St. Louis, and our conversations, for which I am grateful, helped shape my presentation. Finally, I thank Dr. David Newman of the DTP/NCI for providing me with data concerning the project, as well as information on the updated letters in use at present.

ance as to the protocols to be used in making field collections at sites around the world. Each institution was represented by a principal investigator: Dr. Enrique Forero from Missouri, Dr. Doel Soejarto from Illinois, and myself representing NYBG. The three of us had been influenced by the great tropical ethnobotanist, Harvard Professor Richard Evans Schultes, who was, at heart, more Amazonian than Bostonian. His great sense of respect for the indigenous peoples, with whom he spent so many decades, had been passed along to the three of us, as well as to many others he had mentored. We accepted the fact that, without the support, guidance, and participation of the local people that we worked with, our accomplishments would wither. Thus, as the three of us sat around the table with our NCI colleagues, a consensus emerged that we would raise the question of intellectual property and benefit-sharing for the many local people and institutions that would participate in this global search for new therapies.

Up to that point in time, many of those involved in natural products research, as Dr. Norman Farnsworth takes such delight in pointing out, believed that the "tooth fairy" collected and delivered plant samples to their laboratories, ignoring the months and years of relationship building, risky travel and fieldwork, and complicated levels of permits needed to source and deliver plant samples and their associated herbarium vouchers. Given that there were no international treaties governing the collection and transport of plant materials to be evaluated for new therapies at that time, this was not a major part of the protocol being discussed. So, after a few minutes of conversation, Forero, Soejarto, and I decided to make a joint statement to the assembled group of NCI staff. We explained that the political, social, and economic landscape upon which this type of work was undertaken had been changing, and local people, along with their communities and governments, were questioning whether or not any benefits would be provided to their countries and villages as a result of their participation in this hunt for new pharmaceuticals. Providing firsthand reports from the field, we spoke of being compared to those historic figures who would extract resources from faraway places and take them for the benefit of the developed world, where people could afford the cost of anticancer therapies. And we noted how the ethical position of the United States, as the funder of this research, could be undermined by extractive projects such as these, said to be for the public good. I recall the reaction of the larger group as a sort of disbelief at first, not understanding why a group of mere contractors, and field botanists at that, could bring up this issue, when the program was already in place. A somewhat tense discussion followed, and it became clear to the three of us that we were on very thin ice. However, one voice cut through the tension, that of Gordon Cragg. He acknowledged that it might very well be a good idea to rethink the NCI's position on benefit-sharing, and by addressing this embryonic but controversial topic, and indeed leading the way rather than avoiding it, the U.S. and NCI could accomplish a great deal more for the field and, ultimately, the project.

What followed in the many years since that first meeting was an extraordinary effort by Gordon and his colleagues—from many sectors—to craft a benefit-sharing document that would be widely accepted by the diverse parties involved in the NCI's international natural products programs. This was accomplished, with the first version followed by dozens of revisions suited to the particular country a team was working in, as Gordon and the NCI staff circulated drafts to collectors, scientists, policy makers, governmental officials, indigenous peoples, and even critics.

The first documents that resulted from these deliberations were known as the NCI Letter of Intent (LOI) and, in a later version, the NCI Letter of Collection (LOC). These letters were comprehensive, and espoused fairness,

honesty, and transparency in all work that was to be accomplished under the aegis of the DTP. The agreements quickly became the standard not only for NCI's benefit-sharing framework in the dozens of countries in which it worked, but also for many academic and commercial agreements as well. The LOC was executed between an organization in the country where the plants were collected and the DTP of the NCI. The most current letter is presented as Appendix 1 of this paper. In the rich and lengthy dialog that proceeded and has followed the Convention on Biological Diversity (CBD) entering into force in 1993, the NCI program serves as a model mechanism for access and benefit-sharing policies, including building scientific capacity in developing countries. At its inception, the CBD had three main goals: the conservation of biological diversity; the sustainable use of its components; and the fair and equitable sharing of benefits derived from this use. These three themes were spelled out in 42 articles, including Article 8J, which discussed traditional knowledge, innovations, and practices, along with the need for mechanisms for indigenous peoples to share in the benefits arising from the discovery and commercialization of biodiversity. It was no coincidence that the major thrust of the United Nation's CBD paralleled that of the National Cancer Institute DTP's LOI. And I don't think it hyperbole to credit a humble individual who knew that he could accomplish anything in government, as long as it did not matter who received the credit for doing so, with influencing—rather, defining—the shape of the CBD. In the mid-1990s, an updated version of the LOI that acknowledged local capacity in natural products research was developed and used as a deliberate component of the process to source collections and strengthen that capacity.

Despite the fact that the United States was not a signatory to the CBD, there was great interest in the botanical community to move forward with an international agreement that governed our own research, plant collection, and plant exchange programs around the world. Following five years of international workshops involving many dozens of colleagues from botanical gardens, universities, and museums (both large and small), a document entitled *Principles on Access to Genetic Resources and Benefit-sharing for Participating Institutions* was produced in December 2000. This document pledged to "honor the spirit and letter of the Convention on Biological Diversity and laws related to access to genetic resources and associated traditional knowledge and benefit-sharing" with regard to: acquisition of genetic resources; curation, use, and supply of genetic resources; and benefit-sharing, pledging to: prepare a policy to ensure this would be implemented as part of the institutional structure and employ written agreements that were transparent, fair, and constituted best practice. A set of *Explanatory Texts,* comprising 101 pages of sample agreements, definitions, and templates accompany the Principles (http://www.kew.org/conservation/agrbs-policy.pdf). These principles were intended to facilitate the kind of plant collection and research intended by the CBD, and once again, were heavily influenced by the NCI's LOI and LOC. To date, 22 institutions have signed on to the Principles, helping to guide their important international field programs.

I—along with NYBG colleagues Drs. Douglas Daly and Hans Beck, NYBG's principal investigators on these contracts—worked in several of the 18 field sites where our contracts operated from 1986–1996. My personal focus was on Central America and, in particular, Belize. In total we at the NYBG collected 16,276 bulk samples of higher plants for the DTP from 1986–1996, comprising 218 plant families (77% of the families present in the Neotropical region), 1,421 genera (33% of the genera present in the region), and 2,504 species (ca. 3% of the total number described from the region). In Belize, our active collaboration with Drs. Rosita Arvigo and Gregory Shropshire, as well as the

Traditional Healer's Foundation of Belize and many other governmental and non-governmental organizations, resulted in the collection of 2,392 extracts from multiple plant parts, comprising 108 of the 209 plant families, 388 of the 1,219 genera, and 529 of the 3,408 species present in that nation.

FIELDWORK IN BELIZE: THE BELIZE ETHNOBOTANY PROJECT

Belize is a Central American country located on the Caribbean coast, south of Mexico and east of Guatemala. It has a population of 250,000 inhabitants spread over 8,867 square miles, giving a low population density of 28 persons per square mile. Over 70% of the country is under natural forest, and protected areas now cover 36% of the landmass. Despite the small size of the country, its ecosystems are varied and its ethnicity diverse, giving rise to a rich culture with respect to traditional healing. The ethnic diversity ranges from groups of indigenous Maya and the Black Caribs (Garinagu), through the Creole descendants of African slaves, to the more recent Central American and Asian immigrants. Among some of these ethnic groups (especially the Maya and the Garinagu), the use of medicinal plants is spiritual and is linked to myths, rituals, and religion. The knowledge acquired by healers has long ancestral origins.

It was against this backdrop that the Belize Ethnobotany Project was launched in 1988, initially through funding received for plant collecting from the NCI/DTP. The project was a collaborative endeavor involving the Ix Chel Tropical Research Foundation, Belize Center for Environmental Studies, Faculty of Agriculture and Natural Resources of the University of Belize, Agriculture Research and Development Station in Central Farm, The Belize Zoo and Tropical Education Center, Belize Forestry Department, Belize Association of Traditional Healers, Traditional Healers Foundation of Belize, and NYBG.

The most significant goal of the project was to conduct an inventory of the floristic and ethnobotanical diversity of Belize, a country with large tracts of intact forest. The project carried out over 100 collection trips to various locales, and collected over 8,000 plant specimens by the end of 2000. We gathered traditional knowledge provided by dozens of traditional healers and bushmasters of Mopan, Yucateco, and Kekchi Maya, Ladino, Garinagu, Creole, East Indian, and Mennonite descent. Another significant objective was to help foster the teaching and use of traditional knowledge as it relates to plants and the environment. Additionally, the project aimed at promoting conservation of knowledge and biodiversity, through various local initiatives including displays, seminars, post-secondary classes, youth camps, school competition, field trips, and guest lectures. A major output of these local initiatives was the establishment of the first ethnobiomedical forest reserve in the world, dedicated to the conservation of medicinal plants. A more complete discussion of this multifaceted project is presented in Balick and O'Brien (2004).

A program involving short-term, medium-term, and long-term benefits to participants was implemented as part of this project. An example of short-term benefits was the provision of resources to the individuals and the communities in which they lived. Medium-term benefits included formation of the Belize Association of Traditional Healers and later the Traditional Healer's Foundation, which provided royalty payments from the sale of a book on the useful plants of Belize entitled *Rainforest Remedies: One Hundred Healing Herbs of Belize* (Arvigo and Balick, 1993). Over nearly two decades $26,000 (BZ $52,000) was provided directly through biannual payments to the individuals involved in providing information for this book. Details on how the payments helped these individuals to achieve their own goals are provided in Johnston (1998). Today, *Rainforest Remedies* and an-

Figure 1. Gordon Cragg traveling via horseback in a remote area of the Cayo District, Belize, as part of a site visit.

other book published by the project, *Checklist of the Vascular Plants of Belize: With Common Names and Uses* (Balick, Nee, and Atha, 2000), are important documents in support of conservation and ecotourism in the region, as they are rich with information and lore about Belize and its natural resources. In the longer term, the NCI's documents and policies include payments to be made if any of the plants collected under the project achieve commercial status.

Gordon Cragg and our Contracts Officer Elsa Carlton (Figures 1 and 2) had the opportunity to visit the Belize field site in June 1988, and see firsthand some of the results of their work running the NCI plant collections program. During this visit, we gave the most senior of our local healer/collaborators, Don Elijio Panti, a certificate signed by Gordon, expressing thanks for the former's extraordinary collaboration in this project. To Don Elijio the presentation of this document was a milestone in his long life; recognition by a prestigious outside institution that his work had meaning far beyond the boundaries of his small village of San Ignacio. This document, an example of a non-monetary benefit provided by the project, occupied a place of honor in Don Elijio's humble dwelling for the rest of his life, and when patients came to visit, he proudly explained how he was working with NCI on

Figure 2. Elsa Carlton learning by doing—collecting plant samples in the Cayo District, Belize.

an important study. While this might seem a small detail in the history of this important federal organization, Gordon's enthusiasm for providing this type of respect for a local collaborator was a hallmark of the way in which he led the collections program. So it is quite fitting that we return the favor at the William L. Brown Symposium and honor Gordon in the company of so many of his friends.

CONCLUSION

The title of this paper refers to the "Cragg Era" as it relates to biodiversity and natural products research. Prior to that era, there were few rules of conduct for collectors and investigators, and one encountered often limited and confusing systems of permits and protocols in place in source countries, including the United States. Making collections was, in some ways, a sort of a scientific free-for-all, with the game plan often made up on the spot and most usually influenced in some way by the need for efficiency and speed. However, the attitude of governments and their citizens around the world was changing at the time, and in some places biodiversity-related xenophobia had already set in, prohibiting any collection activity, even at times by local people themselves. Lacking a series of international agreements shaped through discussion and consensus building, the search for new therapies from plants could have come to a halt in many areas of the world. At the risk of sounding trite, I, along with many of my colleagues, believe that Gordon Cragg and his co-workers at the NCI set a new standard for natural products research, both in the laboratory and the field. His willingness to consider local partners as equals, and the many years he spent visiting field sites and making people feel appreciated for their collaboration with the NCI will ensure that this type of research can be continued in many critical locales. At a time when the parameters by which we carry out field-based natural products research are changing on an almost weekly basis (through the establishment of new laws and best practices), trust and relationships have become basic requirements for this work. The post-Cragg era will not be an easy one, as the goal of ensuring equity in our partnerships and conserving biodiversity worldwide has not yet been achieved. We who continue to seek an understanding of the diversity, wisdom, and potential of nature owe a great debt of gratitude to Gordon Cragg for his untiring contributions to making this quest possible.

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APPENDIX 1

LETTER OF COLLECTION
Agreement Between
[Source Country Organization, SCO]
and the
Developmental Therapeutics Program
Division of Cancer Treatment and Diagnosis
National Cancer Institute*

The Developmental Therapeutics Program (DTP), Division of Cancer Treatment and Diagnosis ("DCTD"),

*From the National Cancer Institute web site. Available at: http://ttc.nci.nih.gov/forms/loc.doc.

National Cancer Institute (NCI) is currently investigating plants, micro-organisms, and marine macro-organisms as potential sources of novel anticancer drugs. The DTP is the drug discovery program of the NCI which is an Institute of the National Institutes of Health (NIH), an arm of the Department of Health and Human Services (DHHS) of the United States Government. While investigating the potential of natural products in drug discovery and development, NCI wishes to promote the conservation and sustainable utility of biological diversity, and recognizes the need to compensate [Source Country, SC] organizations and peoples in the event of commercialization of a drug developed from an organism collected within their country's borders.

As part of the drug discovery program, DTP has contracts with various organizations for the collection of plants, micro-organisms and marine macro-organisms worldwide. DTP has an interest in investigating plants, micro-organisms and marine macro-organisms from [Source Country], and wishes to collaborate with the [Source Country Government (SCG) or Source Country Organization(s) (SCO)] as appropriate in this investigation. The collection of plants, micro-organisms and marine macro-organisms will be within the framework of the collection contract between the NCI and the NCI Contractor [Contractor] which will collaborate with the appropriate agency in the [SCG or SCO]. The NCI will make sincere efforts to transfer knowledge, expertise, and technology related to drug discovery and development to the [appropriate Source Country Organization (SCO)] in [Source Country] as the agent appointed by the [SCG or SCO], subject to the provision of mutually acceptable guarantees for the protection of intellectual property associated with any patented technology. The [SCG or SCO], in turn, desires to collaborate closely with the DTP/NCI in pursuit of the investigation of its plants, micro-organisms and marine macro-organisms, subject to the conditions and stipulations of this agreement.

A. The role of DTP, DCTD, NCI in the collaboration will include the following:

1) DTP/NCI will screen the extracts of all plants, micro-organisms and marine macro-organisms provided from [Source Country] for anticancer activity, and will provide the test results to [SCO] on an annual basis. Such results will be channeled via Contractor.
2) The parties will keep the test results and subsequently-developed data confidential until approved for publication by the parties. Before either party submits a paper or abstract containing test results for publication, the other party shall have 60 days to review and, as necessary file a sole or joint patent application in accordance with Article 6.
3) Any extracts exhibiting significant activity will be further studied by bioassay-guided fractionation in order to isolate the pure compounds(s) responsible for the observed activity. Since the relevant bioassays are only available at DTP/NCI, such fractionation will be carried out in DTP/NCI laboratories. A suitably qualified scientist designated by [SCO] may participate in this process subject to the terms stated in Article 4. In addition, in the course of the contract period, DTP/NCI will assist the [SCO], thereby assisting the [Source Country], to develop the capacity to undertake drug discovery and development, including capabilities for the screening and isolation of active compounds from plants, micro-organisms and marine organisms.
4) Subject to the provision that suitable laboratory space and other necessary resources are available, DTP/NCI agrees to invite a senior technician or scientist designated by [SCO] to work in the laboratories of DTP/NCI or, if the parties agree, in laboratories using technology which would be useful in furthering work under this agreement. The duration of such visits would not exceed one year except by prior agreement between [SCO] and DTP/NCI. The designated visiting scientist(s) will be subject to provisions usually governing Guest Researchers at NIH. Salary and other conditions of exchange will be negotiated in good faith. Costs and other conditions of visits will also be negotiated in good faith prior to the arrival of the visiting scientist(s).
5) In the event of the isolation of a promising agent from a plant, micro-organism or marine macro-organism collected in [Source Country], further development of the agent will be undertaken by DTP/NCI in collaboration with [SCO]. Once an active agent is approved by the DTP/NCI for preclinical development, [SCO] and the DTP/NCI will discuss participation by SCO scientists in the development of the specific agent.

The DTP/NCI will make a sincere effort to transfer any knowledge, expertise, and technology developed during such collaboration in the

discovery and development process to [SCO], subject to the provision of mutually acceptable guarantees for the protection of intellectual property associated with any patented technology.

6) DTP/NCI/NIH will, as appropriate, seek patent protection on all inventions developed under this agreement by DTP/NCI employees alone or by DTP/NCI and [SCG or SCO] employees jointly, and will seek appropriate protection abroad, including in [Source Country], if appropriate. All resulting patent applications and patents shall be assigned to the U.S. Department of Health and Human Services and managed by NIH. Under current NIH policy, all inventors of such assigned patents may receive royalties in accordance with said NIH policy for any royalty-bearing license(s) for these patent(s).

7) All licenses granted on any patents resulting from this collaboration shall contain a clause referring to this agreement and shall indicate that the licensee has been apprised of this agreement.

8) Should an agent derived from an organism collected under the terms of this agreement eventually be licensed to a pharmaceutical company for production and marketing, DTP/NCI will request that NIH/OTT require the successful licensee to negotiate and enter into agreement(s) with the appropriate [SCG] agency(ies) or [SCO] within twelve (12) months from the execution of said license. This agreement(s) will address the concern on the part of the [SCG or SCO] that pertinent agencies, institutions and/or persons receive royalties and other forms of compensation, as appropriate.

9) The terms of Article 8 shall apply equally to inventions directed to a direct isolate from a natural product material, a product structurally based upon an isolate from the natural product material, a synthetic material for which the natural product material provided a key development lead, or a method of synthesis or use of any aforementioned isolate, product or material; though the percentage of royalties negotiated as payment might vary depending upon the relationship of the marketed drug to the originally isolated product. It is understood that the eventual development of a drug to the stage of marketing is a long term process which may require 10-15 years.

10) In obtaining licensees, the DTP/NCI/NIH will require the license applicant to seek as its first source of supply the natural products from [Source Country]. If no appropriate licensee is found that will use natural products available from [Source Country], or if the [SCG] or [SCO] as appropriate, or its suppliers cannot provide adequate amounts of raw materials at a mutually agreeable fair price, the licensee will be required to pay to the [SCG] or [SCO] as appropriate, compensation (to be negotiated) to be used for expenses associated with cultivation of medicinal organisms that are endangered or for other appropriate conservation measures. These terms will also apply in the event that the licensee begins to market a synthetic material for which a material from [Source Country] provided a key development lead.

11) Article 10 shall not apply to organisms which are freely available from different countries (i.e., common weeds, agricultural crops, ornamental plants, fouling organisms) unless information indicating a particular use of the organism (e.g., medicinal, pesticidal) was provided by local residents to guide the collection of such an organism from [Source Country], or unless other justification acceptable to both the [SCG or SCO] and the DTP/NCI is provided. In the case where an organism is freely available from different countries, but a phenotype producing an active agent is found only in [Source Country], Article 10 shall apply.

12) DTP/NCI will test any pure compounds independently submitted by the [SCG or SCO] scientists for antitumor activity, provided such compounds have not been tested previously in the DTP/NCI screens. If significant antitumor activity is detected, further development of the compound may, as appropriate, be undertaken by DTP/NCI in consultation with the [SCG or SCO].

Should an NCI/NIH patent on an agent derived from the submitted compound(s) eventually be licensed to a pharmaceutical company for production and marketing, DTP/NCI will request that NIH/OTT require the successful licensee to negotiate and enter into agreement(s) with the appropriate [SCG agency(ies) or SCO] within twelve (12) months from the execution of said license. This agreement will address the concern on the part of the [SCG or SCO] that pertinent agencies, institutions and/or persons receive royalties and other forms of compensation, as appropriate.

13) DTP/NCI may send selected samples to other organizations for investigation of their anti-cancer, anti-HIV or other therapeutic potential. Such samples will be restricted to those collected by NCI

contractors unless specifically authorized by the [SCG or SCO]. Any organization receiving samples must agree to compensate the [SCG or SCO] and individuals, as appropriate, in the same fashion as described in Articles 8-10 above, notwithstanding anything to the contrary in Article 11.

B. The role of the Source Country Government ("SCG") or Source Country Organization(s) ("SCO") in the collaboration will include the following:

1) The appropriate agency in [SCG or SCO] will collaborate with Contractor in the collection of plants, micro-organisms and marine macro-organisms, and will work with Contractor to arrange the necessary permits to ensure the timely collection and export of materials to DTP/NCI.
2) Should the appropriate agency in [SCG or SCO] have any knowledge of the medicinal use of any plants, micro-organisms and marine macro-organ isms by the local population or traditional healers, this information will be used to guide the collection of plants, micro-organisms or marine macro-organisms on a priority basis where possible. Details of the methods of administration (e.g., hot infusion, etc.) used by the traditional healers will be provided where applicable to enable suitable extracts to be made. All such information will be kept confidential by DTP/NCI until both parties agree to publication.

   The permission of the traditional healer or community will be sought before publication of their information, and proper acknowledgment will be made of their contribution.
3) The appropriate agency in [SCG or SCO] and Contractor will collaborate in the provision of further quantities of active raw material if required for development studies.
4) In the event of large amounts of raw material being required for production, the appropriate agency of the [SCG or SCO] and Contractor will investigate the mass propagation of the material in [Source Country]. Consideration should also be given to sustainable harvest of the material while conserving the biological diversity of the region, and involvement of the local population in the planning and implementation stages.
5) [SCG or SCG] and SCO scientists and their collaborators may screen additional samples of the same raw materials for other biological activities and develop them for such purposes independently of this agreement.

This agreement shall be valid as of the date of the final authorized signature below for an initial period of five (5) years, after which it can be renewed by mutual agreement. It may be amended at any time subject to the written agreement of both parties. Copies of such amendments will be kept on file at both of the addresses indicated below.

For the National Cancer Institute:

_____

John E. Niederhuber, M.D.
Director, National Cancer Institute

_____

Date

Mailing and contact address:
Technology Transfer Branch
National Cancer Institute at Frederick
Fairview Center, Suite 500
1003 - W. 7th Street
Frederick, Maryland 21701-8512 U.S.A.
Telephone: 301-846-5465
Facsimile: 301-846-6820

For [SCI] or [SCO]:

_____

Name (typed):
Title:

_____

Date

mailing and contact address:

_____

_____

_____

_____

Two-tiered Systems for Access and Benefit-sharing Agreements to Balance the Interests of Source Countries and Researchers

Michael A. Gollin[1,2]

The current regime for research using plant material and associated information imposes very high transaction costs on all involved, including researchers and source country groups providing access to the materials. Generally, under national laws and practices established under the Convention on Biological Diversity, before commencing work, a researcher must obtain informed consent from the source country (including people who control access to the plant material and governmental authorities) and must negotiate an agreement sharing any benefits of the research (an access and benefit-sharing, or ABS, agreement). The consequences of failing to obtain consents are high and rising and include the risk of lawsuits, boycotts, and, increasingly, loss of right to obtain a patent. The principles of ethics and equity underlying ABS agreements are sound, but are at risk of being crushed under the practical difficulties of actually concluding such agreements. It can take months or years to finalize an ABS agreement, and negotiations sometimes crater with no agreement. Many research and source country organizations lack the necessary time, funds, legal and business expertise, and staffing to negotiate ABS agreements. Even sophisticated parties need to invest enormous effort in the process. Organizations providing assistance, like Public Interest Intellectual Property Advisors (www.piipa.org) can help. However, a simpler two-tiered approach would reduce wasted effort and promote the public interest. One example has been tried before, a second is in the implementation phase, and a third remains at the conceptual stage.

The Goal of Balanced Access

As a patent attorney, my focus is on practice, not policy. Pragmatists, like scientists, tend to begin by identifying problems that need to be solved. One of the many problems faced by natural products researchers today is that it is too hard to conclude access and benefit-sharing (ABS) agreements regarding biological samples. The difficulties in reaching these ABS agreements are a real obstacle to further progress both in conserving biodiversity and benefiting from it.

One possible solution is to subsidize the transaction costs, to push through the obstacles that scientists face. A second approach is a two-tiered system, as I will explain. Examples include Gordon Cragg's triangular privity model, the new mechanism under the International Treaty on Plant Genetic Resources, and a modest proposal on e-permits.

We can look at this as a good news/bad news situation. The good news, as Michael Balick and others have noted, includes the development of a regime under the Convention on Biological Diversity (CBD) that satisfies basic principles of equity and ethics by requiring prior informed consent and benefit-sharing as a quid pro quo. This leads people, organizations, and countries to deal with the relationships that flow back and forth with biological materials and the benefits.

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[1]Venable LLP, 575 7th St. NW, Washington, DC 20004, U.S.A.

[2]Thanks to Jim Miller, the Missouri Botanical Garden, the William Brown Center, and the Brown family for honoring Gordon Cragg and for the ongoing contributions they are making to this very complicated and rich field of human endeavor. When Jim asked me to participate in this symposium, I thought immediately of Gordon's role, not as an adventurer, a scientist, and a relationship builder, but rather as a legal pioneer, an innovator in intellectual property, in developing a two-tiered system for benefit-sharing agreements, as a way to balance the many different interests involved in natural products collections.

But the bad news is that the transaction costs and the delays in reaching these agreements can crush opportunities as can the costs of intellectual property management.

One principle to use in solving this problem is the ideal of balance in the public interest. On one hand, the public interest today is generally satisfied by having access to medicines and biological materials. Open access to plants and biological resources helps research happen today. On the other hand, the public interest of future generations may suffer from open access, due to loss of biodiversity, and loss of control by source countries. The principles of ABS agreements, like those of intellectual property more generally, rely on a balance between exclusion and access. Access to innovation allows people to share its benefits, while exclusive intellectual property rights provide people with incentives to innovate for the future. Likewise, a fundamental principle for ABS agreements under the CBD is the right of the source country to exclude people from access to natural resources in order to promote conservation. If biodiversity is destroyed, we won't have the resources to work with in the future.

Balancing Public and Private Interests to Advance Research

Who are the individual stakeholders whose interests need to be balanced? We now take it for granted that source countries have important interests that need to be considered. Researchers in wealthier countries, academic as well as corporate researchers, all have stakes in natural products. There are many stakeholders. These include the bioresource providers, the individuals, groups, and countries, public research institutions, academic institutions, and of course the investors and donors (public and private), small biotech companies, and big pharmaceutical and agricultural technology companies. Generic manufacturers have their interest in commercializing products that have not been patented or whose patents have already expired. For drugs, many other groups have a role to play: doctors, hospitals, patients, public health agencies, insurers who pay for medicines, government agencies that grant patents and give drug approval. For agricultural crops and products, we substitute breeders, seed companies, farmers, food processors, and consumers. This is a very complicated scene with many actors. In some respects this is even more complicated than the science. Coordinating and balancing all these interests is quite a challenging task.

Figure 1 shows a view of the life cycle of a drug. Biological material is just one of the inputs on the left, along with literature, know-how, education, funding, equipment, time, and data. Biological research begins with these inputs and proceeds through applied and clinical research, on to regulatory approval, and commercialization of a proprietary drug. Generic drugs arrive after patent expiration, and eventually the drug dies off or is replaced by another better drug. The research cycle is matched with the steps of the regulatory process and, as shown at the bottom of Figure 1, the patenting process. An invention leads to a patent application, patent prosecution, and patent issuance, follow-on patents on drug formulations, and so on. The illustration shows that many actors and many activities are required to make the transition from a natural specimen to the useful products that make life better for people.

A crucial dynamic in this process involves the relationships and interactions between public and private parties. The interests and obligations of each are interrelated, but they are different. This is one lesson of the Cragg Era—all researchers have obligations toward source countries that provide natural specimens, not just commercial researchers, but academic researchers as well. Universities may collaborate with industry, and (in the U.S.) they license federally funded inventions to corporations under the Bayh-Dole regime. They also transfer inventions to corporate sponsors under sponsored research agreements. However, the

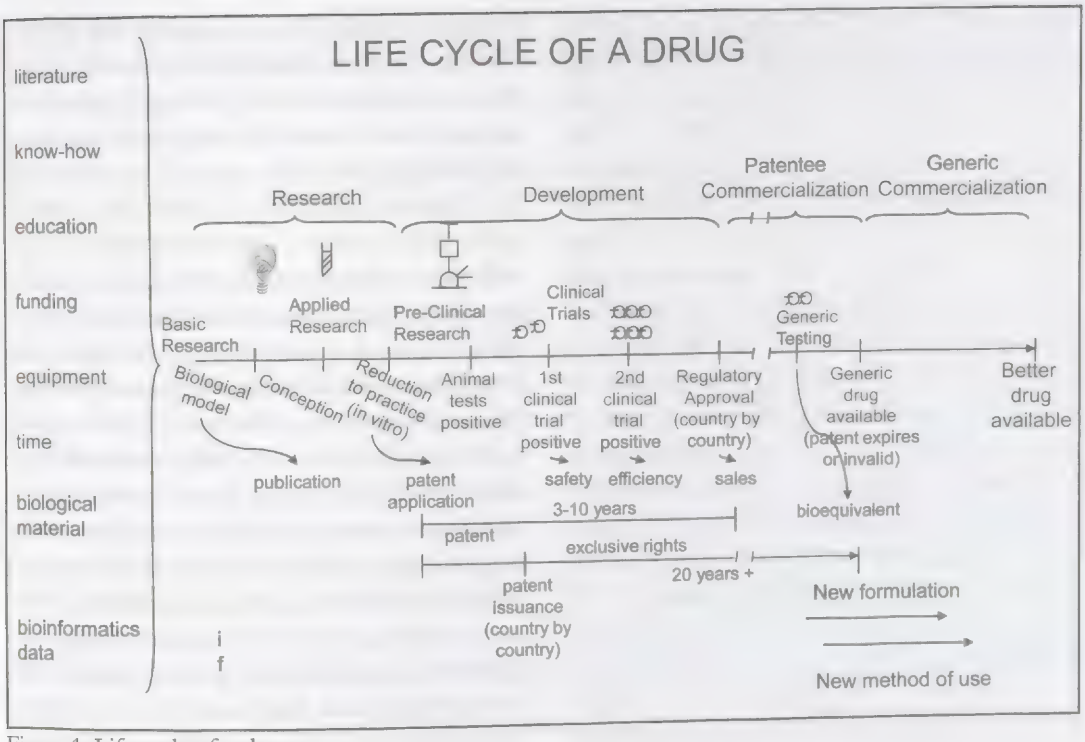

Figure 1. Life cycle of a drug.

great majority of research conducted in universities is ultimately non-commercial or at least non-profit. The same is true for non-profit research institutions that are non-academic, such as the National Institutes of Health, botanical gardens, the Smithsonian Institution, the International Agricultural Research Centers of the Consultative Group on International Agricultural Research (CGIAR), the Venter Institute, and so on.

Non-profit drug companies are a new type of organization; they act like drug companies but don't have shareholders, relying instead on philanthropic donors like the Bill & Melinda Gates Foundation. Examples include One World Health, Medicines for Malaria Venture, Aeras Foundation, and so on. Finally, industry itself does public work, as with Merck's donation of ivermectin to developing countries. Even without making an explicit effort in the public interest, corporate profit-making activities by private industry can benefit the public through extensive investment in research and the production and distribution of new and better drugs.

In my practice of over 20 years, I've had the good fortune to be able to work with all these different types of organizations, public and private. They all share certain interests and innovation dynamics, but there are clear differences as well. This "similar but different" comparison is at the root of the two-tiered system pioneered by Gordon Cragg and his colleagues at the National Cancer Institute (NCI).

I've worked with a wide variety of organizations on biodiversity-prospecting deals. Maurice Iwu's International Cooperative Biodiversity Group (ICBG) grant for Nigeria and Cameroon in 1992 was one of the first bioprospecting agreements that was negotiated outside of the NCI that met modern standards. I worked with Lissy Coley and Todd Capson on the Panama ICBG, with Conservation International on the Suriname ICBG, and Bill Aalbersberg in Fiji.

Traveling to Fiji and formalizing an agreement by sitting on the floor drinking kava kava was a change from sitting in a conference room. I've consulted with Rosita Arvigo in Belize and saw the protected heritage of Don Elijio Panti. I've performed intellectual property work for other developing country organizations, including Chang Mai University in Thailand, several of the CGIAR centers, and nutraceutical companies. This all complements the bulk of my work with pharmaceutical, genomic, and other life science researchers in corporations and research institutions. Although much of the developing country work has been without pay, it has been some of the most rewarding work I've done. And it gives me a unique perspective on the advantages of two-tiered access to biological materials.

THE INNOVATION CYCLE

I developed the concept of the innovation cycle in my recent book, *Driving Innovation: Intellectual Property Strategies for a Dynamic World* (Gollin, 2008), and apply it here to lay a foundation for the two-tiered system of ABS agreements. We can start with accessible knowledge, at the bottom in Figure 2.

Some people refer to accessible knowledge as the public domain, which is not precisely correct, but it helps in understanding the concept. Innovation begins when an individual or a small group has access to available knowledge, or a bioresource, and comes up with a good new idea. That step is creativity. The next step is adoption by the public, in the marketplace, as the creative idea is developed further (as in Figure 1 regarding the pharmaceutical life cycle). Now, many people and organizations are involved, not just one or a few. The third step is accessibility, as the new knowledge enters into the domain of accessible knowledge. This is a general paradigm for all innovation, with intellectual property driving the cycle by selectively restricting and broadening access to new ideas and products.

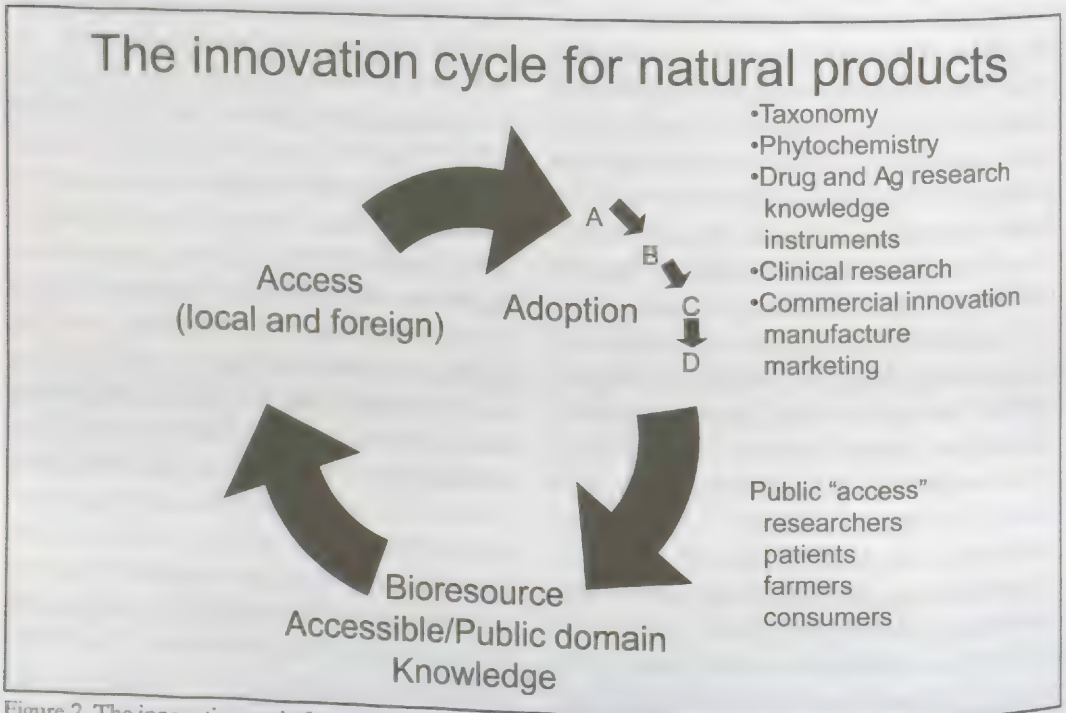

Figure 2. The innovation cycle for natural products.

In the field of natural products research, the plant kingdom and microbial flora can't really be called "knowledge," because they are wild. However, these natural resources do fit the general pattern of the innovation cycle. In this view, the first step is access by local people, who know where the plants are, or foreign people, who come in and find them. The creative step comes as part of research using taxonomy, phytochemistry, drug and agricultural research, scientific knowledge, and instrumentation. The process of development of knowledge and products that can be adopted and used by society continues with clinical research, commercial innovation, and, ultimately, manufacturing and marketing of products. Finally, this cycle leads to public access to the benefits of innovation by patients, farmers, and consumers.

Problems arise when you have blockages in this cycle. One blockage is a lack of access, which stops progress, and that's a major problem. One reason for a lack of access is that the resource has been destroyed. Another reason is that no ABS agreement can be reached. Another blockage occurs when there is no research relationship that can go forward into further development and adoption by society. And finally, the cycle doesn't work well if there is a blockage in the entry of the innovation into the accessible domain, where intellectual property (IP) rights are tangled up and are too proprietary, and so they don't come back into the pool of accessible knowledge for other people to use. That is a very complicated system in a very small nutshell.

Solving the Problem of Fair Access

One approach to improving access to natural resources, and a simple one, is to subsidize the transaction costs. It takes months or years to gain access to new resources. It costs a lot of money. It takes a lot of time. The parties may have to negotiate for years to gain access to a new collecting place. That may be good because it leads to long-term relationships, but during a career, a researcher may only be able to work closely in only one or two countries based on those relationships. And there are a lot of lost opportunities. Meanwhile, the loss of biodiversity continues. From the corporate point of view, with the innovation cycles that we see in pharmaceutical and agricultural interest and bioprospecting, without the natural resources, money will be invested elsewhere, where there are no blockages. Money goes where money can be made, and if you don't have a good business model for bioprospecting, the investment goes into other industries or types of research.

One way to solve this problem is with subsidies. Some philanthropies have decided to pay for the source countries to have counsel and professional assistance to help them implement ABS rules and negotiate ABS agreements. But there is a grave shortage of professionals able to provide such services, and their costs are high.

An alternative to subsidies is to promote voluntarism. This is an approach we came up with in the late 1990s that led to the founding of an organization called Public Interest Intellectual Property Advisors (PIIPA) in 2002. PIIPA was formed partly in reaction to the attacks of 9/11 and the withdrawal of the U.S. from the world, using an international public, non-profit framework to promote private, non-governmental solutions.

PIIPA is a matchmaking organization. There are about 500 international volunteers in the IP Corps who register with PIIPA. Assistance seekers from developing countries ask for help, and there have been about a hundred projects so far. PIIPA matches them up and helps them get assistance with intellectual property projects in a broad range of areas including bioprospecting agreements, trademark, patent, and copyright. PIIPA has received support from the National Institutes of Health's Fogarty Center, the Smithsonian Institution, the Rockefeller Foundation, the Sigrid Rausing Trust, the U.K. Department for International Development, Venable, and the Ford Foun-

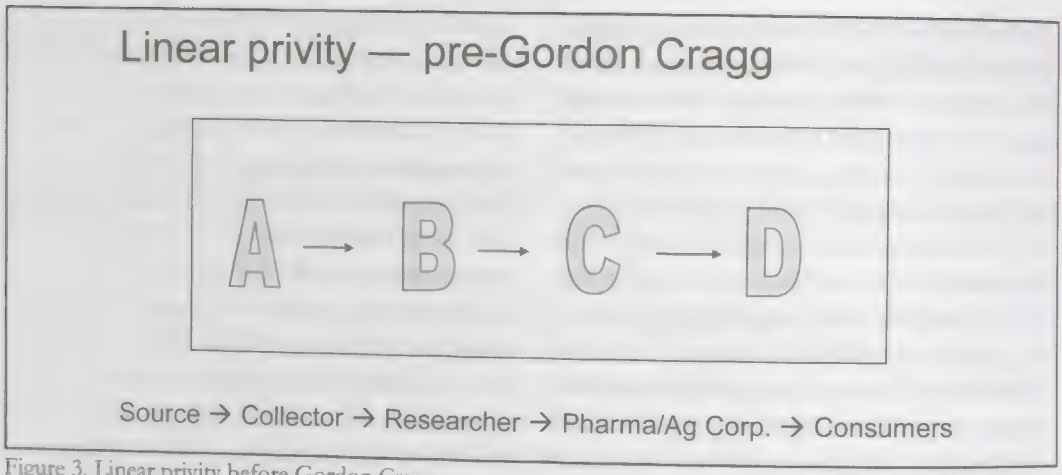

Figure 3. Linear privity before Gordon Cragg.

dation. PIIPA offers help and is always looking for more help. To build the network of IP volunteers and to help assistance seekers apply, PIIPA was set up on the e-commerce model. PIIPA has learned that it can take months or years, and it can take hundreds of hours of professional time, to accomplish the desired result. PIIPA's philosophy is that by providing developing country organizations with access to experienced IP counsel, better and fairer results can be achieved.

TWO-TIERED SYSTEMS

The two-tiered system is a way to reduce the transaction costs of ABS agreements rather than subsidizing them. A pre-Gordon Cragg type of biodiversity access is shown in Figure 3.

The flow of material goes according to this paradigm—from source country to collector, to researcher, to corporation, to consumers, in a linear fashion. Something might flow back to source country "A", but maybe not much.

TRIANGULAR PRIVITY

As Figure 4 shows, triangular privity differs from a simple A → B → C → D flow of rights. In the first step, the source country provides materials to NCI under a simple Letter of Collection (LOC). Under the LOC, some benefits flow

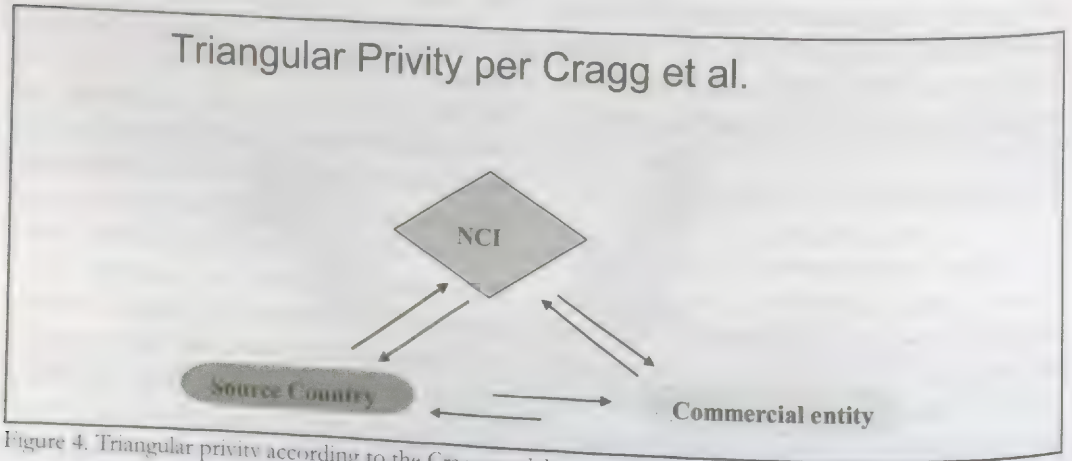

Figure 4. Triangular privity according to the Cragg model.

back, such as research collaboration, data about the flora of the country, and so on. When NCI makes a useful invention, it may then license its rights to a commercial entity for further development, but in this case, under the terms of the LOC, the NCI requires the commercial entity to get permission from the source country. The NCI-corporate agreement is conditioned on the formation of a corporate-source country agreement. The NCI-source country research agreement set forth in the LOC is streamlined because it doesn't have to deal with commercial terms, which really bog down negotiations. The great majority of the material flowing from source country to the NCI has no commercial significance, so triangular privity reduces transaction costs of negotiating an agreement for the few situations that truly matter. In those cases, the corporate partner must negotiate a true ABS agreement with the source country, sharing a portion of revenues, and so on.

A similar concept has moved forward into the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA), which came into force in 2004 after many, many years of negotiation. The ITPGRFA can be seen as a successor biodiversity treaty to the CBD. It is limited only to 64 food and forage crops, but these include some of the most important agricultural plants. These are not as interesting as wild species containing the most diverse chemical structures. As Jim Miller told me, spinach is uninteresting from a phytochemistry point of view because all the interesting chemicals have been taken out to make the plant palatable so we can eat it. But the ITPGRFA model is that breeders and researchers can freely obtain plant genetic material from the multilateral system for public non-commercial use. This may include information exchange, sharing technology, and capacity building.

Commercial use under the ITPGRFA is research that results in restricted rights, subject to intellectual property protections—patented, or secret, or otherwise. In this case, an equitable share of the monetary benefits must be paid, and a portion of that needs to go back to farmers. In agricultural research, farmers are as important as those who conserve wild biodiversity.

The treaty thus establishes a paradigm similar to that of the NCI LOC, in that non-commercial research is easily performed, without the burdensome need for negotiating detailed access and benefit-sharing agreements. It's a fairly simple system for non-commercial research purposes.

The treaty negotiations leading up to the ITPGRFA recognized the difference between private and public research, and that public researchers' first-tier benefit-sharing can be different from the private sector's second-tier benefit-sharing for private research. Although the treaty is hugely important to agricultural research and conservation, the two-tiered nature is worth greater attention from both policy makers and deal makers.

Three sections of the treaty establish the framework of a two-tiered ABS system. Article 12.4 of the ITPGRFA lays out the framework for using a standard form material transfer agreement for public research. The treaty doesn't say exactly what the terms should be. Article 13.2 says that monetary payments should be made in the event of commercialization—but it doesn't say how much they should be or how the amount should be determined. Finally, Article 19.3 provides for the establishment of some kind of a trust account, which will handle the receipt and distribution of payments received under this multilateral system.

Back in 1993, the CBD was a new treaty, too, and the framework for access and benefit-sharing had not yet developed into what is now commonly referred to as ABS. Anybody in this field now understands the range of problems, scenarios, and approaches that come under the ABS heading. Let us look ahead 15 years, then, and predict that the ITPGRFA two-tiered sys-

tem will be in place, administered at the Food and Agriculture Organization, or somewhere else. Hopefully such a two-tiered system that regulates access and benefit-sharing in the agricultural field will help public breeders access the biodiversity they need to achieve improved crops. As with the triangular privity model, the effect is to streamline public use, leaving more complex arrangements to the commercial sector.

Making predictions is risky, and there are many questions that could be asked. Will transaction costs for ABS agreements generally be higher or lower than they are today? Will the need to design standardized non-commercial access agreement forms for agricultural research actually reduce costs relative to custom negotiated agreements? Will the ITPGRFA's multilateral system result in more bureaucracy, more delay, more people involved, higher overhead? Or will the standardization of forms reduce the transaction costs? From the perspective of all public and private and consumers involved, it would be preferable to minimize overhead and standardize things as much as possible.

E-ABS PERMITS: A MODEST PROPOSAL

After considering a) the problem of high transaction costs, b) solutions suggested by the triangular privity model used by the NCI natural products program, and c) the ITPGRFA two-tiered approach, the benefits of a two-tiered system suggest a modern way to improve natural products research. My modest proposal is the establishment of a system of e-permits, and it is fitting to describe it in Gordon Cragg's honor.

The idea of an e-permit or an e-ABS agreement is a concept worthy of broader discussion. Some of the principles of an e-ABS regime are as follows:

- Nations could opt in to the e-ABS regime, if desired, or they could choose not to.
- The e-ABS system could be established by a non-profit, governmental, or international organization.
- The organization would establish the basic terms of use, fees, and governance.
- An e-commerce website would be provided for non-commercial researchers to obtain an e-ABS permit. Basic information would be entered into the system (identity of researcher; affiliated organizations; nationality, location, type and amount of material to be collected; research to be done with the material).
- A basic level of benefits would be set forth in a web-based form, stipulating what the recipient would share. The benefits could be typical of those in ABS agreements over the past decade, including acknowledgment of the source of the biological materials, sharing of information obtained about the material, training, sharing of technology and equipment, research collaboration, and payment of a defined use fee.
- The permittee/recipient of the material would make a commitment not to commercialize innovations resulting from the biological material, without a further agreement. This is the same model as NCI's triangular privity model. (A contractual commitment not to commercialize further is analogous to open-source software licenses, where the recipient of software code promises that any modifications to the code will not be kept secret, but will be shared publicly.)
- To complete the permitting process, a fee would be calculated depending on the number of samples, the researcher would make payment (e.g., via credit card), and an e-permit would be sent to the researcher.
- The permit would be reported to the particular country involved, and the funds would be disbursed to that country.

This system should satisfy the treaty requirements of the ITPGRFA, so as to simplify ABS

transactions for crops, and also transactions for non-crop biological resources. It would also satisfy the principle of full informed consent, because countries could opt in, or not. The greatest advantage of this system is that non-commercial researchers, such as botanists, would be able to obtain a permit online, in real-time, instantly with a click through a button. In theory, it could work just like buying a book through Amazon.

Commercial research, development, and use of the specimens would still require a negotiated "custom" agreement. This should be easier to obtain if you've taken the great majority of the research agreements and put them online. That leaves the source countries with the ability to focus on only the commercially significant ABS agreements. Hopefully, the experience of a streamlined e-ABS system would also yield smoother approvals of commercial ABS agreements. But if a country doesn't want to participate, or for various reasons, if they can't participate, then there's no harm done, and the status quo can continue.

Implementing an e-ABS System

As with any new invention, this system raises several questions. First, some countries don't recognize e-commerce agreements as legally binding. (These are known as "click-through" agreements, where the user reads a contract, and clicks "I agree" before being able to proceed to further screens.) In such countries, various signatures, stamps, signs, and approvals may be required. The need for an agreement to touch all these different hands is not a trivial problem.

Second, this e-ABS regime carries substantial set-up and administration costs. Websites such as Amazon and Google are easy to use, but that is the result of support by hundreds and thousands of very smart and talented people working behind that web page to make it work very easily. Likewise, to establish the e-ABS system would be time-consuming and expensive.

Third, where would the system be housed? At FAO? At the Conventional Biodiversity Secretariat? At some existing or new NGO?

Finally, one must consider the e-ABS system's compatibility with, and interaction with, other ongoing initiatives in biodiversity prospecting. One of these initiatives is the so-called "Designation of Origin," or DOO, movement. Several countries already have DOO requirements. These may include a rule that patent applicants who are working with natural products identify where they got their material from. This is fairly straightforward. Other laws also require the applicant to certify compliance with local ABS rules. That is more complicated. The strictest DOO rules require the patent applicant to provide proof that the material was obtained legitimately, with a copy of an ABS agreement and permit. As a patent attorney, I see great impractical administrative burdens in the stricter DOO regimes. The technical requirements involved in preparing a patent application are extremely time-consuming and complicated. To add further requirements presents a heavy burden both to applicants and patent offices around the world. Indeed, the DOO requirement may be impossible to meet in countries without an efficient ABS regime.

So one benefit of the e-ABS, e-permit idea is that it would make it relatively easy and straightforward to get a permit, thus mitigating the burden of a DOO regime. However, this benefit would vanish if the e-ABS system were not applicable to research that leads to the filing of a patent application. Increasingly, organizations are arguing that the filing of a patent application is in itself a commercial act. An e-ABS permit should be available for investors who file patent applications, particularly in countries with DOO laws.

Another initiative is the idea of certificates or manifests for biological samples. Here, every sample, from the time it is collected and forever, has to have some kind of manifest to show its

provenance. This could be technically impossible or at least extremely expensive to administer, tracking material as it goes from person to person in the innovation cycle.

Finally, universities and research institutions face similar problems of administrative burden and transaction costs in negotiating and signing material transfer agreements (MTAs) for each transfer of material (whether new or modified). Several groups are pioneering electronic web-driven systems for material transfer agreements, to streamline the administrative burden faced by organizations transferring specimens from one to another. Such e-MTA systems are analogous to e-ABS systems and should be encouraged.

Australian e-ABS System

Is it a viable approach to have e-permits or e-ABS agreements? After elaborating the basics of such a system, I discovered that the Australian government has set an online two-tiered e-ABS website. It is part of the Genetic Resources Information Database (GRID) at www.deh.gov.au/biodiversity/science/access/permits.html. In the first screen, you can apply for a commercial or non-commercial purpose. You can provide the requisite information, pay your fifty dollar fee, and receive your permit. This is, then, a two-tiered, e-ABS, web-driven, e-commerce model in operation in Australia.

The Cragg Legacy

To conclude, we can put the two-tiered system in perspective in the arc of history of intellectual property (Gollin, 2008). Confidentiality agreements can be dated back to ancient Egypt, where Irtisen, the chief scribe of Pharoah Mentuhotep, prepared a hieroglyphic stone tablet (Stele C-14) proclaiming that he knew the secrets of hieroglyphics, of making tablets, ink, and papyrus, religious rituals, and so on, and that he promised not to divulge these secrets to anybody at all (with the exception of his eldest son), without the permission of His Divine Sovereign, Pharoah Mentuhotep.

The first patent laws were invented 4,000 years later. In 1474 in Venice, the Venetian Council passed a law defining the scope of patent rights. The law says, more or less, that to advance innovation, we promise that if an ingenious person brings an invention to us, whether they're Venetian or foreign, we will guarantee exclusive rights to that invention, and if anybody copies it, the inventor can destroy it and get paid a penalty of 100 ducats. As a patent attorney, I was amazed to find that every element of modern patent law can be traced to the Venetian Patent Act of 1474.

Moving forward another 500 years to 1981 and to another innovation in intellectual property, the Bayh-Dole Act balanced public, nonprofit, and commercial interests in innovation. The idea that Senator Birch Bayh and his colleagues came up with was that the prior system didn't work, where government owned all inventions that were funded in research at universities, and so they pushed ownership out to the universities, and said that universities must manage the patents and should license them. This change unleashed a whole range of wonderful innovation in and around universities and for the whole world.

Finally, we have the triangular privity model. A key kernel of wisdom here is that there should be an efficient way to mediate between the interests of the source country, typically a poor developing country, and the recipient, typically a technology-rich country.

All these initiatives are the product of political activity and the work of individuals at the right time and place. None of these innovations in new intellectual property structures come without new insights, new ideas, and a fight to put them to work. Gordon Cragg was one of the people who recognized and championed the importance of a two-tiered system of triangular

privity for sharing the benefits of access to biodiversity.

In conclusion, it is difficult to strike a balance between access to bio-resources and restricted access, between access to technology and the exclusivity of intellectual property rights, but we have seen that such a balance is crucial to promote both conservation and innovation. A healthy approach involves a substantive recognition of legal rights and other interests. It also requires procedural efficiency, a point that is too often neglected in policy discussions. We should always think about the practical efficiencies of any particular access system to make sure that it is viable.

By these standards, two-tiered systems stand the test of time. They can streamline research, by allowing non-commercial research to go forward, while helping conserve resources for commercial negotiations. A two-tiered system can be fair and can promote healthy international and personal relationships, as Gordon Cragg's experience shows. A fair, efficient system helps drive the innovation cycle forward, while conserving biodiversity. Thus, the long-term contributions of Gordon Cragg and his team are not just scientific discoveries, and not just human relationships, but also a pioneering long-lasting legal innovation, two-tiered systems of access.

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Envisioning Equality:
Re-imagining the Political Economy of Biological Resource Use

Bronwyn Parry[1,2]

Gordon Cragg's most significant, if under-recognized, contribution to natural products development was to begin the process of envisioning a more effective mechanism for equitably redistributing the profits derived from the collection and use of genetic materials. The multi-tiered access and benefit-sharing agreements that he played a central role in developing and instituting in the 1980s have now become the normative regulatory instrument for governing use of such materials collected under bioprospecting agreements worldwide. It has even been suggested that their use be extended to cover transactions involving human genetic materials. In this paper I provide a brief retrospective assessment of these agreements: their role as a translational instrument of the Convention on Biological Diversity, their efficacy and the extent to which this relies on the science of drug development, and the implications of their prospective future employment in new areas of 21st-century technoscience.

Introduction

In 1985, more than 20 years ago now, Gordon Cragg and his associates at the National Cancer Institute (NCI) and the New York Botanical Garden started what they then considered to be a modest, one might even say relatively private, endeavour. This was to begin, in accordance with their own moral principles, the task of thinking about how to redress some of the inequities that had characterized the collection and use of biological resources to date. There were pressing reasons to do this: the two institutions were about to collaborate in undertaking a new and extensive program of collecting—in some 25 tropical and subtropical countries—for the NCI's natural products development program. They were very cognizant that earlier projects had produced few direct benefits for the countries from which the materials were sourced, and they became concerned to ensure that their program not replicate these injustices. They began to consider how collection work could be undertaken "fairly and justly" so that the communities in which they were collecting would be assured of getting a "decent share" of any profits that might eventuate from their later use. In short, they began to envision how to create and implement a much more equitable political economy of biological resource use.

Their discussions culminated in the drafting of some simple documents. The first of these, the aptly named "Letter of Intent," was a statement of their commitment to invoke a new philosophical and practical approach to the collection process: one that would incorporate for the first time a pledge to share the benefits of the commercial utilization of collected biological and genetic resources with source countries. This document later took on a formal constitution evolving into the more detailed and contractual "Letter of Collection." The concept of "benefit-sharing," which had its genesis here, was quickly embraced by an international community seeking an effec-

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[2]The author would like to thank the William L. Brown Center at the Missouri Botanical Garden and its benefactors for sponsoring her attendance at the symposium, Jim Miller and Joshua Rosenthal for so generously inviting her, Bruce Ponman and Ruth Ann Bizoff of the Missouri Botanical Garden, and her co-presenters for making it such an enjoyable experience.

tive mechanism for the equitable redistribution of profits derived from the exploitation of both genetic resources and indigenous knowledge pertaining to their use. Although it is rarely acknowledged, the benefit-sharing agreements that Gordon Cragg and his contemporaries devised in the mid to late 1980s not only predated the Convention on Biological Diversity's (CBD) later and more sophisticated access and benefit-sharing (ABS) regime, but, in fact, provided the archetype on which this, and all other access and benefit regimes, are based. Agreements introduced under the ABS regime have since become the normative regulatory instrument for governing use of materials collected under bioprospecting agreements worldwide. It has even recently been suggested that their application be extended to cover transactions involving human genetic materials (Simm, 2005; Schroeder and Lásen Diaz, 2006).

It is, perhaps, precisely because these agreements offer what is perceived to be an equitable, a virtuous, one might even say "salvationary" model for effecting redistributive justice that they have been so widely championed. Many, many scholarly articles on bioprospecting and genetic resource use (the most recent published in a special issue of *Developing World Bioethics* in 2006) promote their adoption, accepting often quite uncritically, their ability to alter the fundamental, and historically inequitable, dynamics of natural resource use. The CBD's unwavering commitment to the ABS regime has been forcefully signalled by their decision (as agreed at meetings in Bangkok and Grenada in 2005 and 2006) to universalize the ABS regime to a global scale. Given the depth of international commitment to this regulatory model, it seems more important than ever before to begin the process of rationally assessing its utility.

In this paper I wish to make a small contribution to that endeavour by looking at just one well-publicized benefit-sharing agreement—that involving the San people of southern Africa and the collection and subsequent commercialization of use of the *Hoodia* Sweet ex Decne. succulent, the appetite-suppressing qualities of which were first identified by the San. I don't wish to reiterate every detail of that now long-running case, which has been helpfully summarized in publications elsewhere (Terblanche, 2003; Wynberg, 2004; Wolfson, 2005). Rather, what I wish to do here is to utilize the case as a means of discussing and analyzing how difficulties that both were, and were not, anticipated have acted to problematize the application of benefit-sharing models in practice. In undertaking an assessment of the predicted difficulties of implementing ABS agreements (many of which remain underresearched), there is perhaps no better place to start than by reviewing an analysis on this subject first undertaken by Gordon Cragg himself during an interview that I conducted with him some ten years ago in Maryland.

It was in 1995, exactly ten years after the NCI's first rudimentary benefit-sharing agreements were developed, and ten years ago now, that I first met Gordon Cragg when he agreed to be interviewed for my doctoral study into the rise and operation of the contemporary bio-prospecting industry. In the course of several interviews, Gordon was both generous enough with his time and reflexive enough about his own practice to offer me a cogent and unalloyed assessment of not only the prospective value, but also the prospective shortcomings of the benefit-sharing model. This, I have discovered in retrospect, was relatively unusual, but also entirely characteristic of the man and his honest, forthright, and self-effacing attitude toward his own intellectual legacy and contributions. Gordon's astute analysis provides some key insights into what he considered then to be the primary impediments to the effective operation of ABS agreements. They remain in my view as pertinent as ever before. Some will seem very evident, even obvious. Nevertheless, I would argue that many still remain unaddressed.

However, prescient as Gordon was, he, and indeed few others, could possibly have antici-

pated in 1985 how several other developments (in information technology, branding, marketing, and consumerism) would act to erode the efficacy of ABS agreements in lived experience by the beginning of the 21st century (some 20 years later). Although these developments could hardly have been predicted, I will argue here that they have a greater capacity than any yet identified to fatally compromise the operation of ABS agreements—with very serious consequences for source countries. This paper is therefore structured in two parts. In the first section, I outline (through use of segments of the transcripts of my 1995 interview with Gordon Cragg) just some of the issues that he himself identified as posing particular difficulties for those responsible for implementing and sustaining ABS agreements. Many of these relate to the circumstances of production of commodities derived from collected materials. In the second, I turn to consider a different and rather less well-discussed matter: how transformations, not in the production, but rather in the consumption of products created from collected biological materials are facilitating the circumvention of ABS agreements. In both instances I will use the San-*Hoodia* case to illustrate my arguments. The choice of this case study is entirely arbitrary; the argument could as well be applied to many others—something to consider when assessing the implications of these developments for the future application of ABS agreements globally.

BACKGROUND TO THE SAN-*HOODIA* CASE

While the San-*Hoodia* case is in many ways very complex, it might be helpful to begin by simply summarizing its history and main features. The San peoples of the Kalahari region of South Africa, Namibia, and Botswana have known and used the succulent *H. gordonii* (Masson) Sweet ex Decne. as an appetite suppressant for many generations. In 1995, following nine years of experimentation, the South African state–funded Council for Scientific and Industrial Research (CSIR) was able to isolate the active ingredient, the molecule P57. CSIR was granted the patent on P57 the following year and in 1998 signed a licensing agreement with the British company Phytopharm. Phytopharm then sub-licensed P57 to the pharmaceutical company Pfizer for a reported U.S. $2 million for further product development. It was not until 2001 that the San became aware of these developments and only then as a consequence of a media investigation invoked by several conservation and indigenous rights organizations. As a consequence, a Memorandum of Understanding between the CSIR and the South African San was developed in 2002, which included a commitment to introduce a benefit-sharing agreement (Wynberg, 2004: 856–860).

Under the terms of this benefit-sharing agreement, which was signed in March 2003, the San will, in theory, receive 6% of the royalties that CSIR receives from Phytopharm as a result of the successful exploitation of the products. It is, however, very important to note this is not 6% of the profits derived from sales of patented *Hoodia* products. It is 6% of 10%–20% of net profits that CSIR receives for the duration of the patent. As Wynberg notes (2004: 857), this amounts to as little as between 0.03% and 1.2% of the worth of net sales of the product. The San would also receive 8% of the single payments that CSIR receives from Phytopharm as they reach successive drug development milestones. However, Pfizer subsequently withdrew from their licensing agreement with Phytopharm, not, it is rumoured, because of their unwillingness to be a party to such an agreement, but rather because of their inability to effectively synthesize P57—a matter to which I shall return shortly. In 2004, Phytopharm entered into negotiations with Unilever, and in November of that year Unilever purchased licenses to develop and commercialize P57 as a food supplement. Two years later in 2006 a further ABS agreement was signed between the San and the Hoodia Grow-

er's Association of South Africa in the hope of capturing some of the value that was likely to be produced through their propagation of the succulent for commercial use in these appetite-suppressing products.

The successful negotiation of such ABS agreements is certainly unprecedented in South Africa and represents an important milestone in the struggle to secure economic autonomy for indigenous groups. However, as commentators such as Wynberg (2004) have noted, while these agreements have been widely lauded and promoted as a model for stakeholders in other developing countries to emulate, closer investigations reveal "concerns that impede a wholehearted endorsement" (Wynberg, 2004: 857). In reviewing these concerns, it became evident that many had been pre-empted as early as 1996 by Gordon Cragg and communicated to me in the interview we conducted that year. Despite Gordon's willingness to articulate and reflect on them, and my (and others') interests in publicizing them, they remain largely unaddressed to this day.

I therefore begin the following section by addressing these concerns in turn, using extracts from my interview with Gordon to illustrate not only the issues, but also his mental acuity and prescience in identifying them so long ago. While Gordon was referring in these interviews to concerns that emerged in relationship to benefit-sharing negotiations then occurring between the NCI and other stakeholder groups (including state governments, indigenous groups, non governmental organizations [NGO], and pharmaceutical companies), they remain remarkably pertinent to those taking place today. I then turn in the fourth section to consider a further set of issues that have been much less well anticipated, but which nevertheless have a demonstrable capacity to fatally undermine the efficacy of these ABS agreements upon which the prosecution of the ideals of social justice in bioprospecting have come to rely. I discuss both here as I believe they warrant much more detailed consideration by the scientific and commercial community. They form, as it were, the basis of this morality tale.

SOME ANTICIPATED, BUT LARGELY UNRESOLVED, PROBLEMS WITH ABS AGREEMENTS

Can the State Be Trusted?

"Well of course that is an interesting point and lots of people question that—we tend to deal at a national level with organizations that have strong government links and if any profits were to materialize they would probably go back to the government … but then it's up to the government to disburse it but lots of folks say well gosh these governments in many cases are not going to be having an interest in some little community out there where the thing was originally collected … it's a difficult one. One could make some sort of statement to the government trying to encourage them to make sure some of it gets back to local communities but ways of enforcing that … well. And the Biodiversity Convention, well that all happens at a national level and that's one of the criticisms of it that these groups might be left out of this because genetic resources are considered to be a national resource and while some governments would have a pretty enlightened attitude, others, to be honest, probably couldn't give a damm about what's going on with their ethnic groups."[1]

As Gordon here identifies, and as other commentators on bioprospecting have noted, the CBD has had the effect of "nationalizing" what were once commonly held genetic resources and cultural knowledge. As the now legitimate owners of these resources, the nation state is afforded an unimpeachable right to determine

[1]All extended quotes are derived from two interviews conducted between the author and Gordon Cragg at the NCI Natural Products repository in July 1995 and April 1996.

the terms and conditions under which they may be accessed and used. There is a presumption (oft-stated) that the nation state can be trusted to fulfill their legal and moral obligation under the CBD to ensure that the indigenous groups that provide knowledge and resources to bioprospecting endeavours are "justly" and "fairly" compensated for their contributions. While the ABS agreement that was finally negotiated between the CSIR (a state-funded research institute) and the San Council provides for some monies to be returned to this indigenous group (which of course is welcome), the state, in the form of CSIR, remains the primary beneficiary. For example, it is the CSIR that will retain the vast majority of the royalties from the sale of patented *Hoodia* products and all intellectual property rights in products so developed. Despite having contributed the vital indigenous knowledge about the appetite-suppressing qualities of the *Hoodia* succulent, the San do not share any rights of co-inventorship in patents based on uses they identified.

The CSIR (and by default the sponsoring South African state government) has been retrospectively criticized for developing *Hoodia* to the exclusion of the San. However, they might argue (controversially) that they were simply fulfilling their primary obligation under the CBD: to sustainably develop *Hoodia* (a national resource) for the benefit of all of the State's citizenry, of whom, they might argue, the South African San are but one constituent element. On this basis they could legitimately argue that the South African San will receive benefits from the development of this national resource in much the same proportion as the rest of the South African population. Not all states' governments will want to prosecute this line of argument; some will be perfectly content to make arrangements to provide additional compensation to indigenous stakeholders. However, for those who do not (those nation states which, as Gordon so succinctly put it, "couldn't give a damn about their ethnic groups"), two interrelated aspects of the CBD unwittingly provide ammunition for their cause. The first of these is the primacy that the convention gives to the nation state over indigenous communities as ultimate "owners" of biological resources and associated traditional knowledge. The second, and related, problem is the entirely uncertain and poorly defined legal position that indigenous groups occupy with the CBD. While these groups are recognized under Article 8J and elsewhere as rights-bearing citizens, it is not made clear at any point how exactly their rights are to be balanced with those of other nationals.

The South African state government's relationship to its indigenous groups was undoubtedly informed (in the years in which *Hoodia* was first targeted for development) by the philosophy of apartheid on which many of that State's relations were promulgated. Those who were first involved in the negotiations suggest that this attitude was clearly evidenced in the CSIR's confident assertion that the San tribes had either "disappeared" (as reported by CSIR at the time, and as written up by Barnett, 2001) or were so widely scattered as to be of wholly insignificant number. Whilst the South African state's attitude toward its indigenous groups was rehabilitated in the post-apartheid period (resulting in part in this ABS agreement), these developments illustrate well the difficulty of prosecuting the rights of indigenous groups in bioprospecting agreements in circumstances where the state has no particular interest in upholding or promoting those rights. This is undoubtedly exacerbated by the fact that the state remains, under the terms of the CBD, the "legal owner" of the resources, retaining the right to exploit them in whatever ways they see fit subject to equitability clauses, which are in turn open to interpretation. The matter of how the rights of indigenous stakeholders might be safeguarded in such circumstances has yet to receive sufficient attention from either academics or policy makers.

The Perils of Ethical Paternalism

"If a compound is sent by a supplier and a test is done and some very interesting and novel result comes out of that, then the NCI could claim sole inventorship rights … I have to say that I have a bit of a problem with that myself—but this is the way patent law works … we have had instances of that—in the patent on prostratin, interestingly enough [x—a university-based ethnobotanist collecting for the NCI] is a co-inventor on that, not the actual healer. Now I don't know what the actual reasons for that are, and I would trust [x] implicitly that he has the interests of all the indigenous peoples—the community that he works with there—their interests very much at heart, but [still] the actual healer wasn't the co-inventor."

This raises a second issue that Gordon identified in our interview. Although he didn't characterize it in exactly these words, I think it fair to describe it as "the perils of ethical paternalism." Ethical paternalism occurs when the state or an individual interferes with a person's liberty by making decisions on his or her behalf in order to protect them from a "perceived harm." According to Wynberg, CSIR defended their failure to involve the San in their earliest negotiations with Phtyopharm on the basis that this might have raised the San's expectations of involvement and prospective compensatory return (Wynberg, 2004: 854). Dr. Marthinus Horak, the scientist in charge of the CSIR project at the time, confirmed this in an interview, saying "we always intended to speak to the community at some stage, but we did not believe it would be appropriate to do so before the drug had passed on the clinical tests and been finally approved. We did not want to raise their expectations with promises that could not be met" (Barnett, 2001).

Although the scientists at CSIR were possibly well intentioned, their paternalistic attitude created serious difficulties. Principal amongst these, as Wynberg and others have noted, is that their failure to involve indigenous groups early in the negotiation process—here rationalized by reference to an ostensible desire to protect such groups from the perceived harm of an unfulfillable promise—has, in reality, acted to create a much greater harm: the indigenous groups' fundamental disempowerment from the negotiation and disbursement process. Had the San been consulted earlier, they may have been in a stronger position to claim a co-inventorship right. As it now stands, CSIR is the sole patent holder, and as such is set, theoretically at least, to be the primary recipient of royalties returned through licensed use of these products. The San remain tertiary beneficiaries expected to receive only a very small percentage of any generated profits. As a consequence the San themselves have emerged from later negotiations with a deal that some of their advocates, such as Roger Channels, understandably argue is better than nothing, but which others have suggested is gravely inadequate and restrictive.

The Fundamental Inequities of the Patent System

"Another point is that a lot of these countries don't have the legal expertise to develop these agreements like these high-powered company lawyers—I mean these companies get the best and these folk are single minded in making the best deal for the company—but are some of these poorer groups going to be in the same position to get a good deal?"

As Gordon here notes, while indigenous groups may attend negotiations and express their desires in terms of the use of their collected indigenous plants and knowledge, if their bargaining power is already eroded by late, post-factum involvement, they can only then rely on intermediaries such as NGOs to argue their case on their behalf. These intermediaries, which include lawyers, conservation NGOs, and indigenous rights organizations, are undoubtedly act-

ing benevolently toward the indigenous groups they represent; however, they may also, in some instances, be themselves culpable of a degree of ethical paternalism. This may manifest itself in their acceptance of agreements that they consider are the best that can be negotiated on behalf of the group but which may reflect the particular interests of the intermediaries rather than those of the represented indigenous community. For example, in instances where support funds or operational help for the case were provided by conservation NGOs, the resulting ABS agreements may secure funds dedicated to biodiversity conservation, rather than benefit-sharing, which takes the form of infrastructural provision for, say, indigenous education or health services.

The problem of how to secure appropriate representation for indigenous groups in international ABS negotiations is highly problematic and, in my view, remains largely unresolved. The intrinsic imbalances in the respective power of indigenous groups, the state and state-funded agencies, and large international companies remain very evident. These have been brought into sharpest relief in negotiations over the assignation of intellectual property rights. Even now, few negotiators for developing countries have sufficient knowledge of, or means of active engagement with, global regimes for the international protection of intellectual property to be able to successfully prosecute their claims to patent protection without the pro bono assistance of legal specialists from northern states. It is these specialists, primarily, who have recently been advocating for the introduction of a new methodology for securing interests in patents for indigenous groups that have contributed genetic resources or traditional knowledge to the invention of those products: the disclosure of origin certificate (Dutfield, 2005a, 2005b).

As Dutfield has helpfully outlined, these might take a variety of forms, from a voluntary disclosure to patent examining offices of the source of the relevant constituent elements of the product, to a mandatory requirement for disclosure of the same, to a formal requirement by patent applicants to produce certification that the genetic resources and/or traditional knowledge used in the product's manufacture have been acquired in strict accordance with current ABS provisions. Establishing the "legal provenance" of these constituent elements in this way might, it is argued, more effectively substantiate subsequent claims to rights of co-inventorship or, at minimum, more robust, prosecutable rights to benefit-sharing.

What is not perhaps as clearly assessed is the likely cost-benefit implications of introducing systems of "certificates of origin." In some countries, the apparatus for implementing such a system is already in place as the certification of origin simply formalizes the existence of an agreed compliance with extant ABS requirements by commercial collectors. This, however, raises an obvious, although frequently overlooked, point: if compliance has already been agreed contractually, there is much less need for a certification of origin. Many other countries, though, do not yet even have in place ABS regulations, and the cost of introducing both these and an accompanying system for certification of origin could, as I have noted elsewhere, be exceptionally burdensome economically (Parry, 2004).

As Dutfield and I have also noted, certifications of origin are unlikely to provide an effective means for compensating for the use of genetic resources or traditional knowledge that "originate" in (that is to say, are endemic to, or a cultural artifact of) multiple countries, as claims of origin are so contestable. This is so in the following instances: where genetic or biological resources have been sourced from ex-situ collections or when traditional knowledge on historical use has been culled from published sources; or where the relative contribution of either the traditional knowledge or collected molecular or genetic elements to the finished product is unascertainable or unverifiable. This is the case in many, many instances of bioprospection and drug development.

The development, implementation, and incorporation of these systems into the existing Trade-related Aspects of Intellectual Property Rights (TRIPs) agreement is also likely to entail, as Dutfield notes, "a high cost" (Dutfield, 2005b: 3). Moreover, as he goes on to argue, such systems are only likely to "be relevant to a tiny proportion of income-generating life science products … [they] may offer little practical benefit to any national economy or population." There is a serious risk that a requirement to introduce "certification of origin" protocols will result in developing countries committing scarce but precious resources to the implementation of yet another highly bureaucratic and costly system for tracking usage of collected genetic resources and traditional knowledge, one that, like existing ABS regulations, provides an infrastructure for compensation but ultimately precious little by way of financial returns. This raises then, finally, one last key concern that Gordon raised in his analysis: the dangers of an overreliance on royalty payments as the primary compensatory mechanism in benefit-sharing agreements.

Royalties, Royalties Everywhere and Not a Drop to Drink …

"You know these royalties are very much overplayed: the chances of discovering something that is going to be a so-called billion dollar drug are actually infinitesimally small, and unfortunately a lot of countries think there is a great pot of gold there and it just isn't going to happen, so I think the short-term benefits especially in terms of helping them to improve the capabilities of universities or a group like INBio to carry out their own work will be very important."

The difficulty with royalties, as Gordon here so perceptively noted, is this: they are always prospective. In other words, royalties are only secured when the speculative value of the "product" is realized in the marketplace. In pharmaceuticals and neutraceuticals, this in turn is reliant on successful drug or product development and, crucially, on maintaining, through patenting and licensing, the exclusivity of the marketed products. As Gordon here noted from long experience, the probability of all these conditions being met is infinitesimally small—there are so many points in the chain of development and production where successful commercialization can fail or indeed as I shall illustrate in just a moment, where exclusivity can be fatally undermined or circumvented. Although this argument has not always been embraced, Gordon very wisely in my view counsels that indigenous groups should attempt to secure as many immediate, non-pecuniary benefits—education, training, and general capacity building—as they are able in the short term as a safeguard against the non-realization of royalty returns.

It is evident that in the San case most emphasis was placed on securing a proportionate share of prospective royalties. As Wynberg (2004: 857) notes, although "the importance of non-monetary benefits has been widely recognized over the past decade," in the San case there was only "a nominal agreement to provide bursaries to assist with education." She goes on to say that, moreover, "in practice it has been acknowledged that in the haste to finalize the benefit-sharing agreement, the alternative [non-monetary] benefits took a back seat to the negotiation of royalty shares." Very interestingly, Wynberg asserts that the decision to prioritize royalties over other forms of non-monetary benefits may have been part of a deliberate strategy on the part of the San to exercise their autonomy in decision-making by securing funds, the prospective use of which they alone were then at liberty to determine, thus resisting forms of ethical paternalism that would have seen negotiators nominate "suitable" types of use or infrastructural development.

In order to understand why this overreliance on royalties has proven already to be a very high-risk strategy, we need to move in the last part of this paper to consider some very important but largely unanticipated developments

in natural products research and marketing that have more power than anything I have yet identified to fundamentally destabilize the benefit-sharing agreements that all these protagonists have so carefully engineered.

SOME UNANTICIPATED PROBLEMS WITH ABS AGREEMENTS

In 1996, when I first began my investigations into bioprospecting and benefit-sharing, many of the people I interviewed asked why I was undertaking the research at that time. As one person put it to me: "why now, have they found something really big?" The successful development, patenting, and marketing of a natural product that might provide an income stream for the nation and indigenous communities from whom it was sourced has always been the Holy Grail of contemporary bioprospecting, the benchmark by which the success of the entire enterprise would ultimately be judged. In *Hoodia* we seemed, at first, to have found the Grail.

As those who have read my book *Trading the Genome* (which is the outcome of that research) will know, I am skeptical of the power of existing ABS agreements to deliver the returns they promise. This is not because they are not well intentioned, but rather, I believe, because product development technology will always outrun the signatories' capacity to keep track of the use of natural products. In my book, I argued that what is of primary value in collected biological and genetic resources is the information that is either embedded in, or attached to, natural products—information about the molecular or genetic composition of the active compounds or indigenous information about their applied use. Both, I argue, can be readily detached from the corporeal material itself and used in isolation from it.

Information of all types can be very easily up and downloaded, circulated and replicated in a global economy. It is, thus, very difficult to trace where this valuable, derived information goes and how it is successively used. Indigenous information and knowledge about medicinal plant use are, for example, increasingly being placed on publicly accessible databases or widely disseminated in other ways, often through the Internet. Drug development science similarly relies on biochemical, molecular, or genetic information now frequently utilized in isolation from the biological materials in which it was originally embodied. Without a means of effectively tracing all these subsequent uses that are made of these kinds of extracted information, there is simply no way of securing adequate compensation for the communities that provided them in the first place. This problem is a fundamental shortcoming of the royalty-based compensatory form: it is impossible to pay a royalty unless you know to whom that royalty should be paid. That can only occur when it has been possible to effectively trace both the producers and all successive consumers of the resources in question.

While these difficulties are significant, they have, in my view, been profoundly accelerated in recent years by a further set of developments, a phenomenon that, although largely unanticipated, nevertheless is already having very serious implications for the workability of existing ABS agreements. I am going to call this phenomenon "speculative bio e-commerce." Its adverse impacts are nowhere more evident than in the *Hoodia* case, which has so far promised so much by way of a successful outcome for ABS agreements. So what is speculative bio e-commerce, and why is it having such a detrimental effect on the application of ABS agreements? An analysis of the global marketing of *Hoodia* products is very educational in this regard.

Information about the appetite-suppressing qualities of *Hoodia* has been available since it was first divulged by the San to scientists interested in the plant's properties. This indigenous information has since been supplemented with anecdotal, but nonetheless very powerful, additional

information about the performance of *Hoodia*, or more specifically its active ingredient P57, in subsequent clinical trials. All of this information has together combined to create a buzz of excitement about *Hoodia*, a potent mythology about its prospective value as a weight loss aid.

What I think has been entirely unanticipated by either ABS stakeholders or commentators is the commercial value that this zeitgeist information could create in the world of e-commerce. There has always been an unwritten belief amongst those involved in bioprospecting that consumers won't pay for natural products unless they have scientific verification of their effectiveness and safety. Clinical trials and patents are assumed to provide the necessary mechanisms for securing this sort of quality control. Only products that have successfully passed through a robust and extensive regime of clinical trials, safety assurance, and tests of efficacy are deemed to meet agreed scientific and regulatory performance standards. It is assumed that consumers value this endorsement so highly that they will reject products that have not successfully passed through this process of qualification, ensuring that the patent holders' monopoly (for patents on pharmaceuticals are only offered when these standards are met) on production, licensing, and sale of the product remains intact, or at least largely unrivalled. This, however, has not proven to be the case.

If you go onto the Internet now and type in "Hoodia," literally hundreds and hundreds of pages of advertisements will appear for *Hoodia gordonii* products. These products are being offered for sale by e-commerce suppliers of natural products—all of whom are capitalizing on the buzz that has been generated around *Hoodia* production and use. For although many of these suppliers claim that their products have been "independently tested" or "certified," they have not, in fact, passed through the rigorous test of efficacy and quality assurance required for patented pharmaceutical and nutraceutical products.

Nevertheless, people, it seems, are prepared to buy these products even in the absence of proofs of efficacy and safety that were once considered non-negotiable prerequisites for consumption. Why are they doing this? I would argue that they do so because what they are actually buying is not the product per se, but rather the speculative, almost allegorical information that has been generated about its prospective value as a weight loss product. In other words, they are consuming it as what Nikolas Rose (Rose, 2006) calls a "promissory technology." Consumers have no means of establishing with certainty either the provenance or the efficacy of these so-called *Hoodia* products, and indeed the vast majority of them are reported to contain almost no active ingredient. However, this seems to matter surprisingly little to them. Just as individuals are now prepared in greater numbers than ever before to buy "prescribed" pharmaceuticals over the Internet, from Canada or other countries, with no guarantee that they are not part of a huge and burgeoning global trade in counterfeit drugs, so these consumers are also willing to sacrifice formal evidential proofs of efficacy and safety in the interests of immediate gratification of desire.

This has, though, profound implications for the operation of access and benefit-sharing agreements and, in the *Hoodia* case, for CSIR, Phytopharm, and ultimately, of course, the San. It is in this case deeply ironic that much of the enthusiasm that surrounds *Hoodia* and its effectiveness as a weight loss supplement has been generated by the circulation of leaked (and thus unsubstantiated) information about the success of preliminary tests of efficacy being currently conducted by CSIR and Phytopharm as part of their long commitment to securing a defensible patent on *Hoodia* and its derived products. At present, however, these patents have yet to be secured. The e-commerce market is cleverly anticipating the success of those patents and feeding off the speculative frenzy that accompanies

rumors of their impending issue. E-commerce traders are of course also selling aggressively right now in anticipation of the constraining impact that the grant of patents (with their associated rights of exclusivity in marketing and sales) will inevitably have on their ability to operate in what is now a completely unregulated cyber-market.

But what of the San in all of this—how, if at all, do they benefit from this burgeoning online market in *Hoodia* products? The unpalatable answer is that they don't. Because the San only have a benefit-sharing agreement with CSIR—and as CSIR and Phytopharm are still bringing their products through the necessary stages of patented drug development and do not yet consequently have any *Hoodia*-based products on the market—none of these products or the millions and millions of dollars that are currently being generated from their sales will ever be channelled back to the San.

It has been suggested that this situation might nevertheless be productive for the San in the long run in two ways—firstly, because it will increase interest in the patented products when they do come to market and, secondly, because in the interim it will increase demand for *Hoodia* in its natural plant form. There are, however, some difficulties with these arguments: firstly, if individuals buy and use an untested and ineffective product they may well be dissatisfied with it. Widespread discontent may result in a dramatic decline in consumer confidence in the efficacy of all *Hoodia* products, and this could seriously damage the future market for properly trialed and patented *Hoodia* products. Secondly, hype is important for stimulating interest in products, but it is by its very nature transient; demand for a product is very high when it's the next best thing but can collapse dramatically when it becomes yesterday's news. It is deeply ironic and unfair that the San are, at present, unable to profit in any way from the hype surrounding these products during this incredibly important window of opportunity. Some e-commerce suppliers of *Hoodia* have reportedly approached the San with the intention of establishing their own benefit-sharing agreement with them, but have been unable to do so as the San are prevented under the terms of their agreement with CSIR from entering into ABS agreements with any other companies in order to protect CSIR and Phytopharm's (theoretical) exclusivity on production.

Lastly, it has been suggested that the increased interest in *Hoodia* products generated via Internet exposure will at least increase demand for raw *H. gordonii* and that this in turn will increase opportunities for cultivation in situ from which the San might tangentially benefit. However, there are considerable difficulties involved in growing *H. gordonii* on a large scale as it is a desert succulent and not an agricultural crop. While synthesization might provide a more cost-effective method of production, it has been rumored that Pfizer pulled out of the Phytopharm agreement when they found they could not achieve synthesization in a cost-effective way. It is interesting to note that the quantities of raw *Hoodia* that would be required to produce all the products that are currently being advertised on the Internet is so great that it would require vast tracts of South Africa to be under cultivation to theoretically meet the need, which clearly they are not.

Conclusion

So what can we conclude from this analysis of the past and current difficulties of operationalizing ABS agreements, and what do they suggest about how the operation of such agreements might be refined to achieve greater effectiveness? A number of important insights can be gleaned. Firstly, there remain some quite considerable difficulties with the practical application of ABS agreements. While many of these were identified more than a decade ago, they are still not taken into sufficient account when such

agreements are first negotiated and drafted. Issues relating to the role of the state, the representation of indigenous groups, the need to avoid ethical paternalism while still assisting indigenous groups in securing equitable outcomes, all remain prominent, and largely unresolved. In light of the developments that I have laid out in the second half of this paper, the respective weight that ought to be given to royalties and immediate, up-front benefits also needs to be given very careful consideration in the future. For while the assignation of co-inventorship rights and the promise of associated royalty revenue payments are seductive, the probability of assuring income streams for indigenous groups through these mechanisms is comparatively poor.

There can be no clearer evidence, in my view, of how new technologies can act to erode the legitimate chain of disbursement of royalties for use of natural products than that provided by the robust growth of a burgeoning e-commerce market for spurious *Hoodia* products. It could be argued that these suppliers are not even really circumventing or disrupting the establishment of the trail of provenance in *Hoodia* that we know it is essential to maintain if royalties are to make their way back to the San. I say this because in many ways it could be argued that these Internet suppliers are not actually selling *Hoodia* products, for in many instances, the products contain little or no *Hoodia*. They are, rather, selling the idea of *Hoodia*—the speculative mythological value of *Hoodia*.

That, I would argue, is a market in which regulation is going to prove to be very difficult to implement. While regulating the marketing and purchase of desire seems an impossibly difficult task, the practical arguments put forward by Michael Gollin elsewhere in this volume on the need to develop (at least) mechanisms for regulating these forms of e-commerce, as well as alternative calls to commit user countries to take actions against providers who utilize resources acquired in contravention of overarching CBD principles, are clearly worthy of further investigation.

As I have discussed, one mechanism for achieving these aims involves voluntary or mandatory disclosure of origin. One of my primary concerns, though, about disclosure of origin and related "proof of acquisition" protocols is that they assume that it will be easy to establish what it is that is being acquired and capitalized on. I hope that the example that I used in the last section of this paper on the sale of *Hoodia* products on the Internet illustrates the reverse: that it is, in fact, often very difficult to establish in what exactly the value of these products lies. For we can clearly see from this case study that the value that is realized from selling so-called *Hoodia* products on the Internet lies not in the product itself, for often the product is fake and thus has no inherent value as an appetite suppressant. What the emergent and flourishing e-commerce market in *Hoodia* products and supplements demonstrates is that value, in this case, is derived from selling people little more than a promise about the product's prospective worth. However, even though the value of these products is therefore often speculative and promissory, it is nevertheless clearly realizable as capital, as these burgeoning markets in spurious *Hoodia* products show.

The dilemma for regulators lies in the fact that what is being marketed is, arguably, not an effective natural product, but rather just the promise of one. And as regulations stand at present there are no laws against marketing a promise. How it might ever be possible to regulate a global economy in seductive, promissory ideas, products, and technologies is one I shall leave the reader to contemplate.

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Transdisciplinary Team Science and Lessons from the International Cooperative Biodiversity Groups[1]

Flora N. Katz[2*] and Joshua P. Rosenthal[2]

A cornerstone of Gordon Cragg's approach to natural products drug discovery is international, multi-disciplinary, and equitable collaborations. Due to his influence, in part, this philosophy has imbued the NIH-NSF-USDA–funded program, International Cooperative Biodiversity Groups (ICBG), since its inception in 1993. The ICBG Program supports an integrated set of goals in natural products discovery, biodiversity conservation, scientific capacity development, and equitable access and benefit-sharing through such partnerships. The partnership networks operate across countries, institutions, sectors of society, and professional disciplines. Indeed, the ICBG approach of collaborative team science was a pioneering effort that has been followed, more recently, by a surge of transdisciplinary team science initiatives that are emerging from a number of funding agencies. Some broader lessons about international multidisciplinary and transdisciplinary team science can be derived from an analysis of the ICBG history. These lessons address, among other issues: the benefits and costs for science, for conservation, and for participating stakeholders; the features of collaborative research and development projects that contribute to sustainability and productivity; and measures of success.

Introduction

Over the past ten years, multidisciplinary and intersectorial teams designed to address large science and biomedical problems, or assembled in response to public health emergencies, are becoming more common and are having large impacts. For example, the transformative field of genomics grew out of collaborations between molecular geneticists, computer scientists, and engineers, and has benefited substantively by the cooperation (and sometimes rivalry) between the public and private sectors. In global health science where the research often directly engages the public, impacts national policies, and serves national priorities of the host country, an additional element of these partnerships is the communities themselves (Parkes et al., 2005). Where the communities are actively engaged in the research enterprise, the multidisciplinary research team becomes "transdisciplinary," working at the interface of science and society (Choi and Pak, 2006).

Natural products drug discovery is a natural arena for transdisciplinary approaches. The scientific enterprise of drug discovery in itself requires a variety of expertise to conduct the initial collections of materials from the wild, sometimes involving traditional knowledge of local peoples, through biological assays, chemical isolation, medicinal chemistry, animal studies, clinical trials, and in some cases a return to the field for harvesting or cultivation of raw materials. The modern ethical context of natural products research brings yet another complex domain into drug discovery research. The communities and polities from which the materials are collected have an important stake in the research from the outset, one that is increasingly regulated under the United Nations Convention on Biological Diversity (CBD, 1992). Consequently, the political and social landscape must also be addressed thoughtfully in a research project.

This paper considers the rationale, benefits, and challenges of these broader transdisciplinary approaches to drug discovery in the context of the 15-year history of the International Cooperative

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Biodiversity Groups (ICBG) Program (Rosenthal et al., 1999; Rosenthal and Katz, 2004).

BACKGROUND

In the late 1980s a sort of epiphany was reached in the scientific and policy communities about the interrelatedness of several previously distinct concerns. There emerged an appreciation of how little of the world's biodiversity was described or understood (Wilson, 1988), the magnitude and rate of biodiversity loss around the world (Myers, 1988; Wilson, 1992; Ehrlich, 1994), the crucial role that biodiversity played in providing the raw material for new pharmaceutical and biotechnological advances (Farnsworth, 1988; Schweitzer et al., 1991), and the lack of scientific capacity in the developing world, where most biodiversity is found, to explore and use it sustainably (NRC, 1992). When considered together by leading thinkers, a call emerged for win-win-win solutions to these interrelated problems, both in the literature (Wilson, 1988; Reid et al., 1993; Grifo and Rosenthal, 1997) and in the policy community (CBD, 1992; WRI, IUCN, and UNEP, 1992; Gollin, 1993).

The ICBG emerged from that same milieu and is arguably the most successful, best sustained, and most ambitious attempt to address the aforementioned concerns in an integrated way. The concept for the ICBG Program was developed in 1991 at a United States government interagency meeting convened by the Fogarty International Center (FIC) of the U.S. National Institutes of Health (NIH) (Schweitzer et al., 1991). The meeting participants considered the interdependence of bioexploration for new drugs from nature, the preservation of the biodiversity that such exploration depended upon, and the need for incentives to conserve biodiversity, particularly in the face of short-term economic drivers that favor its destruction. Since the greatest biodiversity is largely found in low- and middle-income countries (LMIC), including some of the poorest nations on earth, this latter consideration was critical. Would there be an advantage in addressing these interrelated issues through one integrated program?

Since no single U.S. government agency had a congressional mandate broad enough to support all of these goals, several institutes and centers (ICs) of the NIH, the National Science Foundation (NSF), and the U.S. Agency for International Development (USAID) came together to offer a joint competitive grant solicitation. Based on FIC's international research experience and broad mandate, FIC assumed overall management of the program on behalf of all the sponsors.

The first competitive five-year grants were awarded in 1993, and there have been three rounds of awards to date. USAID withdrew from the program in 1996 due to budget constraints. The following year the Forest Service and the Foreign Agricultural Service of the U.S. Department of Agriculture (USDA) joined the sponsors. Currently eight NIH ICs, NSF, and USDA co-sponsor the program (Table 1), which supports seven cooperative agreement awards in nine countries (Table 2). Total U.S. government funding for the program in 2007 was approximately $6 million. The Department of Energy (DOE) and the Cooperative State Research, Education, and Extension Service of the USDA have agreed to join the program as sponsors of the most recent call for proposals.

To address the complex goals of the program, the grantees assemble teams from the U.S. and partner LMIC institutions spanning diverse sectors and disciplines. Team members frequently include systematists, ecologists, chemists, molecular biologists, database managers, anthropologists, ethnobotanists, lawyers, development experts, and economists. Sectors represented must include a U.S. not-for-profit institution partnering with an LMIC not-for-profit institution as a minimum, but often also involve other academic and government institutions, industrial partners (pharmaceutical, botanicals, and agrochemical), nongovernmental organizations (NGOs), and local

Agency	Period of Participation	Program Interest
National Institutes of Health		
Fogarty International Center	1993–present Administrative lead, financial support	Global health, international collaborations, scientific capacity building
National Cancer Institute	1993–present Technical and financial support	Anticancer agents
National Institute of Mental Health	1993–present Technical and financial support	Agents for mental disorders
National Institute of Allergy and Infectious Diseases	1993–2008 Technical and financial support	Anti-infective agents
National Center for Complementary and Alternative Medicine	2003–present Technical and financial support	Ethnomedical remedies
Office of Dietary Supplements	2003–present Technical and financial support	Botanical analysis
National Institute on Drug Abuse	1997–2003 Technical and financial support	Anti-addiction agents
National Institute of General Medical Sciences	2003–present Technical and financial support	Novel bioactive molecules and new chemistry
National Science Foundation		
Division of Environmental Biology	1993–present Technical and financial support	Biodiversity inventory and conservation
Division of Ocean Sciences	2003–present Technical support	Marine biology
Division of Cellular and Molecular Biology	2003–2008 Technical support	Microbial diversity
U.S. Agency for International Development	1993–1996 Technical and financial support	Biodiversity conservation and capacity building
U.S. Department of Agriculture		
Foreign Agriculture Service	1997–2003 Technical and financial support	Sustainable use of biodiversity, crop protection
Forest Service	1997–2003 Technical support	Sustainable use of biodiversity, conservation
National Institute for Food and Agriculture	2008–present Technical and financial support	Sustainable use of biodiversity, crop protection, and animal health
U.S. Department of Energy		
Office of Biological and Environmental Research	2008–present Technical and financial support	Biological sources of energy conversion and CO_2 assimilation
National Oceanographic and Atmospheric Administration		
Oceans and Human Health Program	2008–present Technical and financial support	Marine biotechnology toward human health, biodiversity conservation

Table 1. Participating funding agencies.

communities. While these teams often comprise public-private partnerships, those partnerships are driven by the applicants with only general thematic guidance from the government. The grantees assemble the teams and negotiate agreements to govern the partnerships and any potential products, following guidelines established by the sponsors and consistent with international and national law and precedent, as well as the specific needs of the contracting parties. They also must develop and obtain prior informed consent (PIC) from the host governments or communities as it

Project (Principal Investigator)	Organismal Focus	Principal Institutions Involved
Biodiversity Conservation and Drug Discovery in Madagascar (David G. I. Kingston)	Tropical plants and marine organisms	Virginia Polytechnic Institute and State University Missouri Botanical Garden Conservation International Madagascar National Centers for Pharmaceutical, Environmental, and Oceanographic Research Eisai Research Institute Dow AgroSciences
Bioassay and Ecology Directed Drug Discovery in Panama (William H. Gerwick)	Tropical plants; endophytic fungi; marine algae, microbes, and invertebrates	Smithsonian Tropical Research Institute Scripps Institution of Oceanography University of Panama Oregon State University National Secretariat for Science, Technology and Innovation (Panama) Novartis Institutes for Biomedical Research Dow AgroSciences
Biodiversity of Vietnam and Laos (Djaja Djendoel Soejarto)	Tropical plants	University of Illinois at Chicago Purdue University Traditional Medicine Research Center (Laos) Vietnam National Institutes of Biotechnology, of Ecology and Biological Resources, and of Chemistry Cuc Phuong National Park (Vietnam) Tam Dao National Park (Vietnam) Bristol-Myers Squibb
Conservation and Sustainable Use of Biodiversity in Papua New Guinea (Louis R. Barrows)	Tropical plants, marine and terrestrial microorganisms, and fungi	University of Utah Smithsonian Institution University of Papua New Guinea Natural History Museum, London National Forest Research Institute (PNG) PNGBioNET Wyeth Research

Table 2. Current ICBG projects, 2003–2008.

is defined by the host government in the context of the CBD and by the principles of the ICBG program for equity and transparency. These latter efforts, agreements and permits, have emerged as major hurdles to biodiversity research around the world and frequently take six months to two years to finalize.

Why Engage in Transdisciplinary Research? Is All This Effort Worth It?

Transdisciplinary research agendas are ambitious and not without risk, but there are significant benefits to the sponsors of the research, the teams of researchers who engage in these activities, and the communities with whom they interact.

For the interagency funding sponsors of these broad programs, common core activities support diverse missions and represent shared economies. For example, the upstream expenses are shared for developing teams, permits, and agreements and getting the teams to remote locations to make collections. Similarly, a biological collection of a given organism can be used for a variety of purposes. For example, the same sample used for bioinventory and conservation planning (NSF) can also be used in bioassays to look for new drugs and dietary supplements (NIH), agrochemicals (USDA), and agents to produce biofuels or for use in bioremediation (DOE). Products from the forest or the sea can be used to create alternative economic activities

Project (Principal Investigator)	Organismal Focus	Principal Institutions Involved
Building New Pharmaceutical Capabilities in Central Asia (Ilya Raskin)	Temperate plants, endophytic fungi, and terrestrial microorganisms	Rutgers University University of Illinois, Urbana-Champaign Kyrgyz Agricultural Research Institute Kyrgyz National Botanical Garden and Biology-Soil Institute of National Academy of Sciences of Kyrgyz Republic Tashkent State Agrarian University (Uzbekistan) International Institute for Study of Avicenna Heritage and Pharmacology (Tajikistan) Tajik State National University Tajik State Medical University Ministry of Agriculture and Environment Protection of the Republic of Tajikistan Kazakh National Medical University Al-Farabi Kazakh National University Institute of Microbiology and Virology (Kazakhstan) Association for the Conservation of Biodiversity of Kazakhstan Eisai Research Institute Wyeth Research
Discovery of Natural Product–based Drugs from Costa Rican Biota (Jon C. Clardy)	Endophytic fungi, lichens, marine actinomycetes and cyanobacteria, marine invertebrates	Harvard Medical School National Institute of Biodiversity (Costa Rica) Broad Institute of Harvard and MIT University of Michigan Novartis Institutes for Biomedical Research
Exploration, Conservation, and Development of Marine Biodiversity in Fiji (Mark E. Hay)	Coral reef organisms and marine microbes	Georgia Institute of Technology Scripps Institution of Oceanography University of the South Pacific Bristol-Myers Squibb

Table 2 (cont.). Current ICBG projects, 2003–2008.

to prevent the natural environment from being destroyed while providing livelihoods for local human inhabitants (USAID). All of these activities can be used for training and developing scientific capacity in the host countries (FIC/NIH) and, together with potential revenues generated from either commercializable products or sustainable-use enterprises, contribute to the benefit package that makes participation by source country organizations desirable.

The grantees also benefit from these more complex funding arrangements. As a cooperative agreement, the government sponsors have substantial interaction with the teams and provide expertise, resources (contracts and materials), oversight, and guidance through a Technical Advisory Group. Formal association with multiple agencies provides the potential for grantees to develop useful relationships that may make possible the future sustainability of individual components of the projects.

Expanded goals can also mitigate risk for all stakeholders. Drug discovery is a necessary but low-probability undertaking that has a high return when successful. Early in the discussions around the CBD, it was thought that biodiscovery from biodiversity would fairly quickly provide new, commercializable products that would return royalties to the source countries as the benefit of engaging in biodiscovery collaborations. This notion rested on unrealistic expectations that have in some cases created significant dissatisfaction and disappointment with the actual outcomes of the research programs (Macilwain, 1998; ten Kate and Laird, 1999). Although commercially oriented biodiscovery for new phar-

maceuticals is still among the most frequently cited arguments for conserving biodiversity, in practice, the expectation of windfall profits is often more a barrier than a benefit to realizing a successful bioexploration/conservation project. Probability estimates for finding and developing a drug from a compound or extract in nature vary widely, but the unambiguous fact that the sum total of the world's biotechnology and drug development research activities have produced fewer than ten natural product–derived FDA-approved new chemical entities (NCEs) per year since 2000 (Newman and Cragg, 2007) makes it clear that this is a relatively rare event. What's more, the vast majority of these NCEs are based on compounds that were originally isolated from nature ten to 30 years previously. Despite these daunting numbers, based on our conversations with industry partners and from viewing the literature, we are currently experiencing a resurgence of interest in natural products in both the industrial and academic sectors. Although the payoff from new structures may occur well into the future, and the rate of finding new structures is much greater today than in the past, for communities and governments looking to justify and support conservation efforts based upon revenue from its immediate use, this low-probability return is not a compelling incentive. Exposing extracts to a wider array of both therapeutic and other (e.g., agricultural or bioenergy) assay areas may increase the probability of finding a useful agent, enhancing the probability of return on the investment for all stakeholders while sharing the risk. More importantly, embedding the discovery effort within other activities that may produce more immediate benefits to the community has clear advantages both as incentive and for equity. This broader and more nuanced approach also produces diverse and enriched information products (see below), investment in future benefits (via training and development of infrastructure), and translational products such as outreach for conservation and impacts on policy.

Partnerships with pharmaceutical and agroscience companies, while not required by the program, have been a component of many ICBGs. Fluctuating interest in natural products within the pharmaceutical industry (Butler, 2004; Koehn and Carter, 2005) has created some challenges, including the unexpected termination of some of these partnerships in midterm. Nonetheless, these collaborations can have substantial benefits for the projects. Pharmaceutical and biotechnology companies use high-throughput screening in rotating state-of-the-art assays that are often not feasible or even available in academic laboratories, affording greater exposure of the extracts to a diversity of therapeutic screens. Because natural products are generally bioactive molecules to begin with, they are occasionally turned into drugs with only modest chemical modification (taxol being the most well-known example) (Wall and Wani, 1995). However, most natural products that become drugs do so by providing a lead structure for substantial chemical modification and semi- or complete synthesis (Clardy and Walsh, 2004; Koehn and Carter, 2005). As a discovery program, the ICBG supports initial chemical isolation and optimization of bioactive compounds, but cannot support the extensive modifications and preclinical and clinical testing required to move a lead down the long pipeline toward a drug. The pharmaceutical partners provide the expertise and substantial financing (DiMasi, Hansen, and Grabowski, 2003) needed to undertake that work, and they provide a drug development pathway, at least for those leads that will have sufficient (developed country) markets, should they be approved. Leads in areas of neglected diseases, on the other hand, do not have these advantages and may move forward in development if championed and supported through other not-for-profit organizations, such as the Medicines for Malaria Venture, the Drugs for Neglected Diseases initiative, or the Global Alliance for TB Drug Development, which typically are not partners at the earlier discovery phases of the ICBG projects. Industrial partners frequently

also provide up-front compensation or milestone payments to the host country, as well as equipment and training, which supports the scientific capacity building and community development or conservation goals of the partnerships.

EMERGENT OUTCOMES

In addition to mitigating risk, cost-efficiencies, and facilitating lead development, a major goal of transdisciplinary approaches is the potential impact of outcomes that emerge from an integrated project but transcend the activities of its components. The team functions as a single advocate that puts all the pieces together and allows each of the parts to support the multiplicity of goals. Research becomes linked to implementation, basic science supports community and policy outcomes, and the community can contribute to the basic science. In many cases, the resulting whole is greater than the sum of the parts. This is illustrated by three examples from the ICBG program.

Development of Sustainable-use Enterprises to Conserve Scientific Resources and Provide Community Benefits in Exchange for Scientific Access: The Fiji ICBG

Discussions around access to genetic resources struggle with the concept of adequate compensation to communities that contribute knowledge or resources to biodiscovery projects. While local communities value their natural resources and would often like to conserve them, use of these resources may be a critical component of their livelihoods. The ICBGs have worked with local communities to develop a variety of models that address the tension between use and conservation and create sustainable economic alternatives to destruction of these assets.

One promising model for coral reef use and conservation has been developed by the Fiji ICBG, a partnership among the Georgia Institute of Technology, the University of the South Pacific (USP), Scripps Institution of Oceanography, and Bristol-Myers Squibb (Table 2), led by Mark Hay. The major goals of this consortium are to discover drug candidates from marine organisms of the coral reefs of Fiji, to develop the South Pacific Center for Biodiversity Conservation and Drug Discovery at USP, and to contribute to the conservation of Fiji's natural resources through partnerships with Fijian communities.

The coral reefs in Fiji are largely owned or controlled by the coastal communities (Aalbersberg, Tawake, and Parras, 2005). In addition to fishing, many communities derive additional income from selling pieces of live coral to the international aquarium trade. (Aquariums use living coral in tropical displays to assist in mineral balance and add authenticity.) Fiji is one of the largest suppliers of live coral in the world and exported approximately 800,000 kg in 2001. However, harvest of live coral is a major contributor to degradation and loss of coral reefs (CRA, 2005). During a planning phase of the ICBG, the team approached the Tagaqe village to negotiate research collections from their reef. Together they developed a proposal for a pilot project to work with Walt Smith International (the largest company in Fiji involved in the export of live coral) to produce an alternative product called "cultured live rock." The ICBG purchased 5,000 pumice blanks from Walt Smith, which the villagers suspended in the water above the reef. Over a period of eight months, during which they halted all collections of natural coral, red coralline algae and coral reef organisms invaded the pumice blanks to produce an imitation coral that looks like and has many of the properties of live coral (filtering water, neutralizing fish waste, and maintaining pH balance). This "cultured live rock" was sold to the new Georgia Aquarium in Atlanta, Georgia (Sanders, 2005). The aquarium, one of the world's largest, publicized the purchase to highlight reef conservation, thereby drawing additional attention to the issue of reef loss. The village kept

half of the revenue from this first successful harvest and used the other half to purchase a new set of blanks to produce a second year's harvest. The ICBG team is measuring the ecological impact of this intervention to ensure that perpetuation and scale up of this practice will not harm the reef. The training Walt Smith and the ICBG team provided to the village in marine aquaculture may prove useful later in developing a sustainable supply of raw material should the scientists find a promising compound from a reef-dwelling organism. The scientists, meanwhile, have proceeded to collect coral reef organisms and test them in a variety of bioassays, in partnership with the village and with USP, and under a separate set of access and benefit-sharing agreements. This has already resulted in the isolation of interesting bioactive compounds, including a group of novel macrolides with potent anticancer activity (Kubanek et al., 2005, 2006). Taken together, this transdisciplinary approach is producing new science, benefits to the participating communities, and conservation of the source of both new scientific discoveries and sustained livelihood for the villagers. Other villages have shown interest in converting from live rock to cultured live rock as a means of protecting their own reefs. While it remains to be seen whether this practice will be both sustainable and scalable, the success of this program has already become part of Fijian national policy discussions with respect to reef conservation, and thus a win-win-win situation for biodiversity, science, and communities.

Development of a National Conservation Initiative to Conserve Scientific and Biodiversity Resources and Provide Economic Opportunities: The Panama ICBG

The ICBGs support conservation outcomes through a variety of activities. These include training in biodiversity sciences, scientific research that contributes to biodiversity management, in situ and ex situ conservation projects, environmental education and outreach, and integrated conservation and development projects such as the one described above (see also Rosenthal and Katz, 2004).

To meet the challenges of an integrated ICBG project, the Panama ICBG (Table 2) chose to focus a portion of their scientific and conservation activities in an area of unique marine and terrestrial biodiversity with high species endemism in the Gulf of Chiriquí on the southwestern coast of Panama (Glynn and Mate, 1997; Ibáñez, 2001; Guzman, Guevara, and Breedy, 2004; Capson, 2006). The area is included within the Tropical Eastern Pacific (TEP), an isolated marine region with possibly the highest regional marine endemism in the world (Capson, 2006). When the ICBG team began working there in 2000, Coiba Island and the surrounding island and marine ecosystems were weakly protected by a legal resolution designating the area as Coiba National Park. However, the imminent closure of the penal colony on Coiba Island and weak enforcement of protection for the surrounding waters newly exposed the area to commercial development, tourism, and overfishing.

With the permission of the Panamanian government, the ICBG began by expanding the exploration and documentation of the park's terrestrial plants, endophytes, and marine organisms. Preliminary surveys of the park uncovered some species new to science and others hitherto unrecorded in the zone (Ibáñez, 2001; Diaz et al., 2005). Collections from the island and surrounding waters yielded bioactive compounds in therapeutic areas important to Panama, including parasitic diseases (Gutiérrez et al., 2005a, 2005b, 2006). Most of the bioassays and some of the chemistry were carried out in Panama in laboratories developed through the program (Kursar et al., 1999; Capson, 2007), contributing significant new research capacity and offering training opportunities to Panamanian students. The team also supported an economic impact analysis of the potential effects of conservation

on the fishing villages bordering the area and did extensive outreach and education activities (Capson, 2007).

In cooperation with other interested groups in Panama, this evidence of scientific importance and potential economic, public health, and conservation benefits to Panama was presented to the Panamanian Parliament. The Parliament subsequently passed a series of legislative acts regulating fishing, designating the area a "National Park under Law," and then expanding the protected area to include a larger region within the Gulf of Chiriquí to protect migrating pelagic fish and marine mammal routes within the TEP zone. The park and surrounding Special Zone of Marine Protection together encompass over 2,701 km^2. With this in hand, the Panamanian government, working with ICBG scientists, requested the United Nations Educational, Scientific, and Cultural Organization (UNESCO) to inscribe Coiba National Park as a World Heritage Site, a designation that was conferred in July 2005 (Capson, 2007). As one of only approximately 160 sites worldwide, World Heritage status provides enormous international prestige and is a source of great national pride. It will bring new tourism and economic benefits to Panama, including the communities surrounding the park, while preserving the biodiversity of the area for future scientific research. These results represent significant scientific, capacity building, and conservation outcomes that would likely not have occurred on this scale without the integrated goals demanded by the ICBG program.

Defining Prior Informed Consent of Local Communities for Genetic Resources and Traditional Knowledge: The Peru ICBG (1994–1999)

The ICBG has acted as a laboratory for policy and procedural experiments with regard to access and benefit-sharing, as well as PIC, and has created an evidence-base in each of the countries in which the team has worked. They have acted as brokers, advocates, and informants for the creation of national policy in response to the CBD. As mentioned above, this area has been a lightning rod for international attention to bioexploration projects. While the legal, social, and political discussion around access and benefit-sharing is often considered an unwelcome burden by natural scientists, the importance of participating in development of functional and equitable models should not be underestimated. Current and future international research involving collected non-human biological and genetic samples for all kinds of research is dependent upon developing such models and a supportive policy environment. In the context of transdisciplinary science, the structure of relationships with academics, governments, companies, and community members enormously influences not only access conditions but also the types of questions asked and the quality of the information that emerges. Ultimately, it is crucial to success.

The Peru ICBG began to navigate these complex issues in 1994, when there was very little formal guidance on PIC from the anthropology community, the CBD, national governments, or indigenous societies. The Peru ICBG was a partnership among Washington University, the Universidad Peruana Cayetano Heredia of Peru, the Universidad San Marcos of Peru, indigenous Aguaruna communities, and the Searle-Monsanto Company, with Walter Lewis as the principal investigator. The project aimed to integrate biodiversity conservation and community development with the development of new therapeutic agents against a wide range of infectious and chronic diseases of relevance to both Peru and the U.S. The partners focused their research on tropical plants, frequently selected using ethnomedical knowledge from traditional healers among the Aguaruna peoples of the Alto Marañón region of northern Peru.

The project quickly ran into controversy—who represented the Aguaruna peoples and could give permission for sharing their traditional knowl-

edge? And who should benefit (Rosenthal, 2006)? A temporary moratorium on drug discovery activities was imposed on the project by the government sponsors while these issues were sorted out. The indigenous federations, international lawyers, scientists, and government officials were brought together to try to resolve these conflicts. Over a period of a year and a half, workshops and meetings were held, and a new model for how to address communal knowledge in this complicated environment emerged from this slow and participatory approach (Greene, 2004; Lewis and Ramani, 2007; Rosenthal, 2006). A series of agreements resulted from the process, including a biological collecting agreement, a know-how license for use of traditional knowledge, a commercial license option agreement, and subcontracts with the Peruvian universities. Lewis and Ramani (2007) discuss these agreements and the negotiations that produced them in more detail. Completing these agreements enabled the project to resume research and capacity building, yielding an array of biodiversity and biochemistry outcomes (Lewis et al., 1999; Rojas et al., 2006). Furthermore, these groundbreaking agreements were subsequently used in the development of Peruvian national laws on genetic resources and traditional knowledge (Ruiz, 1997; WIPO, 2000; see http://www.lclark.edu/org/ielp/peruenglish.html for the English translation of the legislation that emerged). The lessons learned have been widely studied in Peru and internationally.

Other ICBGs have grappled with similar issues and are making significant progress on working with host governments to produce clear guidelines and laws. Whether this emerging legal environment effectively supports transdisciplinary research that engages communities, universities, and others is still to be determined.

Challenges of Complex Partnerships and Diverse Stakeholders

There are many challenges to these diverse and nontraditional partnerships for both the sponsors and the grantees. How does one measure success when there are such broad goals? How should credit be assigned? What are the trade-offs in synergies versus productivity? Is a different type of leadership required for a transdisciplinary team, where the leader is likely to be expert in only one of many areas required for the project? In intersectoral collaborations, each partner represents an individual and often contrasting operating culture, with different needs, widely varying rates of change, and diverse expectations (see Table 3). How can teams balance and reconcile academic, industrial, NGO, and community needs to maximize cooperation? How can they maximize sustainability of outcomes when the grant ends?

When working with low-income countries, we must add to this list of generic transdisciplinary challenges an additional list of hurdles projects must face: shifting political landscapes, meager resources at host sites, few highly trained personnel, national cultural differences, unrealistic expectations, and significant communication barriers to managing a large consortium.

These are big issues, to which entire schools of thought in leadership, business, and international development are devoted. Perhaps the most relevant observation that we can make from the history of the ICBGs is that projects planned with substantial and substantive involvement of all the members of a team are often the ones best able to find compromises that subsequently enable them to sustain working relationships, laboratories, or collaborative projects after a grant terminates. A planning process that takes real advantage of the expertise (and limitations), missions, and related investments of its partners, but effectively builds an exciting new joint endeavor, motivates ongoing efforts to work optimally and productively over time.

While we do not have space to address all the challenges mentioned above, two merit particular attention here. The trade-off between productivity and quality of products, and mea-

	Motivation	Information Management	Time Lines	Decision Making
Academic	Curiosity	Publish	Relatively flexible	Independent
Industrial	Income	Secrecy until product	Narrow	Hierarchical
Non-Governmental Organization	Issue	Constant outreach	Flexible	Variable
Civil Society	Complicated	Free-for-all	Short attention span	Individual and delegated; political

Table 3. Cultural and operational differences among sectors.

suring success vis-à-vis expectations, are two that the ICBGs contend with regularly.

PRODUCTIVITY/QUALITY

The management and communication burden of complex transdisciplinary projects, as well as the frequent need to wait on the work of partners before one can take the next step on a given project mean that, for some scientists, fewer specific outputs per period of time emerge from their work than may come from a more focused project. For example, although bioinventory is an important goal of all ICBGs, a project focused on collections alone is likely to produce a larger inventory in the same funding period. However, as discussed in a previous section, the shared costs and use of the bioinventory results may be ultimately more cost and time effective for the sponsors and for the project overall.

In addition, a transdisciplinary project, such as an ICBG, is likely to achieve substantially richer information content per individual biological specimen. It is self-evident that the value of a collected biological specimen is greatly enhanced by having information on its bioactivity, traditional uses, chemical composition, associated microbes or symbionts, conservation status, and potential economic uses (e.g., therapeutics, crop protection agents, biofuels, industrial enzymes), whether your principal interest is systematics, biochemistry, commercial development, or conservation. The impact of such a specimen will be greater over time—referred to in more publications and perhaps picked up for further analysis and development by others much more frequently (Rosenthal et al., 1999; S. Miller, pers. comm.). The diverse interests and expertise within a single team may ensure such an outcome. For example, with an ethnobotanical specimen collected through a broader and more integrated program, such as the ICBG, there is an increased probability that the information will be acted upon and further explored because the means to carry out bioactivity and chemical analysis is present in the same team that collected the information and contributes to the achievements of that group collectively. The key to achieving these sorts of synergies among components of a program is greater integration of major activities, in contrast to the parallel structure of independent, focused projects with narrower expectations.

MEASURING SUCCESS/MANAGING EXPECTATIONS

Bioexploration, or biodiscovery, is no different than most scientific endeavors in that the overwhelming majority of projects make incremental contributions to our body of knowledge; a very small number make major breakthroughs that are truly transformative for a field or a given application. However, because of the popular profile of "bioprospecting for blockbuster drugs" and the hype that surrounds it, projects like the ICBGs regularly contend with the expectation that they will produce a "new Taxol" (Beattie et al., 2005). These "inflated expectations" affect many aspects of biodiscovery/exploration proj-

ects, like the ICBGs, from funding to data sharing, community participation, collection permits, and contract development.

What is an appropriate expectation and measure of success for an integrated bioexploration program? Below we outline some of the indicators that we have used to assess the ICBGs and summarize progress in each of these areas over the 14-year history of the program.

Organisms examined biochemically: These include approximately 12,000 species, 80% of which are plants, with the remainder divided among endophytic fungi, insects, marine and terrestrial bacteria, and marine invertebrates.

Molecular diversity uncovered: Approximately 1,400 bioactive molecules have been isolated, of which approximately 350 are new to science. Approximately 20 molecules are currently considered active leads for potential new therapeutics.

New species: While more difficult to attribute because of the long delay in species' descriptions, at least two dozen new species of plants have been or are being described, and hundreds of new microorganisms have been analyzed, with public genotype depositions in GenBank.

Scientific publications: ICBG projects have produced over 500 publications, including 350 peer-reviewed articles and 13 books.

Protected areas: The ICBGs have played a major role in the founding of three new protected areas, including one new World Heritage Site (see above), and have contributed to strengthening the scientific management basis for at least nine others.

Training: Over 4,000 individuals have received training in biodiversity and pharmaceutical sciences, with approximately 350 in long-term (one year or longer) programs, resulting in 135 Master's and Ph.D. degrees. About 70% of these trainees come from developing countries, the rest are young U.S. scientists.

Institutional capacity: Several dozen developing country organizations in 15 countries have been substantially upgraded as research institutions.

Policy: At least seven participating countries have made policy or legislative changes based, in part, on ICBG lessons. The Working Group on Access and Benefit-sharing of the CBD regularly looks to the ICBGs for case studies and data to inform their discussions.

Co-investments: Beyond U.S. government support, over 50 different organizations, including host country governments, foundations, and seven different pharmaceutical and agroscience companies, have made direct investments in trust funds or research capacity in host country institutions through the ICBGs.

While it is difficult to identify a comparison group for transdisciplinary projects, it is evident that there have been substantial outcomes in many areas in the ICBGs. These include the principal outcomes sought in traditional-focused drug discovery projects—novel bioactive molecules that are leads for potential new therapies. Nonetheless, it is possible that there may be fewer drug candidates in the development pipeline at the end of the program than would be the case for comparably sized but more focused and less ambitious projects. We will need more time and data to assess how this integrated approach compares in that regard.

Whatever the outcome may be in regard to drug candidates in the near to medium time frame, the outcomes for transdisciplinary projects such as the ICBGs should be evaluated as a package, since that is what the groups are requested to conduct—an integrated program with a package of outcomes. For the host countries, the package of benefits developed through these transdisciplinary projects are clearly diverse, and many are likely to yield sustainable achievements. The benefits assembled through an ABS agreement include the possibility of a drug or commercializable product, but also include valuable deliverables that are more assured and more immediate (Coley et al., 2003; Kursar et al., 2007). The development of scientific ca-

pacity or the conservation of natural resources, for example, can provide significant self-perpetuating returns. The translational products are also more diverse, such as drug candidates, laboratories, reserves, and policies. Training young and senior scientists in the context of a research project is likely to produce among the most important and longest-lasting benefits of all and deserves special attention.

Implications and Conclusion: Future of Transdisciplinary Science

The issues that inspired the creation of the ICBG 15 years ago are even more urgent and cogent today: the alarming rate of biodiversity loss (Mace et al., 2005) and the importance of biodiversity as a source of new medicines and biotechnology (Hallock and Cragg, 2003; Newman, Cragg, and Snader, 2003; Beattie et al., 2005; Tan, Gyllenhaal, and Soejarto, 2006; Newman and Cragg, 2007). The need for clarity and paradigms in the policy community around CBD concerns also remains pressing. The issuance of the Bonn guidelines in 2002 (Secretariat of the Convention on Biological Diversity, 2002) was an important contribution in this regard and was partially informed by the ICBG experience. Considering these policy and equity issues, there will be a continual need for transdisciplinary approaches in the area of bioexploration and, indeed, in an increasing number of biomedical projects that engage communities and depend on public participation in research. Moreover, there is a growing awareness that the most important problems in both basic research and in global health require collaborations that cross traditional disciplinary boundaries. In light of this, it is worth considering whether funding agencies are prepared to support this type of science, and whether universities will have mechanisms to train the next generation of scientists in these models.

The NIH and many in the research community have recognized the need to support these broader research ventures. One way to assess the trend is to look at grants awarded over time. From the inception of the ICBG in 1993 through 2006, there was an absolute increase of 3.6-fold in the number of NIH grants recorded in the public CRISP database that included the words "multidisciplinary," "interdisciplinary," or "transdisciplinary" in the title or abstract. Such grants represent 6.0% of total awards in 2006. In fact, the NIH is moving progressively toward administration and funding models that can accommodate such research. One notable example is the trans-NIH RoadMap Initiative (http://nihroadmap.nih.gov/), which provides five-year experimental funding for programs that crosscut institute missions and includes a special theme on Research Teams of the Future. The NIH has also recognized the need to give equal status to collaborators from different disciplines or different institutions by introducing the option of designating "multiple Principal Investigators" on NIH grants (http://grants.nih.gov/grants/multi_pi/). Even the U.S. Congress has recognized this gap and addresses the need for interdisciplinary research in authorization language for NSF (http://science.house.gov/legislation/leg_highlights_detail.aspx?NewsID=1773) and the congressional reauthorization for the NIH (http://www.nih.gov/about/director/crsrept.pdf). The Fogarty International Center of the NIH, with its broad mandate in global health, has long advocated such multidimensional approaches in programs such as the ICBGs, Health and Economics, and, more recently, the Framework Programs for Global Health (http://www.fic.nih.gov/programs/training_grants/framework/index.htm). All of these measures recognize that many research projects are already operating in this mode and producing powerful scientific outcomes. While not replacing investigator-initiated, more focused projects, NIH and other funding organizations will need to be flexible enough to include mechanisms that can capture these new, innovative relationships.

Similarly, universities are reconsidering their traditional departmental structures to provide opportunities to train their students in team problem solving that crosses departmental boundaries and to support faculty who wish to teach and do research in these crosscutting areas (Macilwain, 2007; Nature, 2007). These new models include public-private partnerships, interdisciplinary centers, and joint degrees or new tracts within traditional degrees.

In our view, complex programs such as the ICBG contribute to a more holistic, realistic, systems approach to health research. While drugs and vaccines are clearly major tools to promote health, we will always be fighting a losing battle if we do not both develop and deploy these tools in a framework that promotes economic stability, food security, and the conservation of the natural resources and ecological integrity that protect us from disease and contribute fundamentally to our well-being (Daily, 1997; Scholes, Hassan, and Ash, 2005). This can and should be done both through focused research projects that are planned and conducted with such a framework in mind and through larger transdisciplinary projects that seek to integrate diverse objectives into coordinated efforts that yield transcendent outcomes.

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The Role of Natural Products in Future Drug Development

Nature's Potential: How Many Drugs Could Come from Plants?

James S. Miller[1,2]

Nature has traditionally been a rich source of therapeutically useful chemical compounds. Large scale efforts to systematically screen plants and other organisms in the later part of the 20th century, however, yielded fewer new compounds with pharmaceutical potential than had been anticipated. The size and present state of knowledge of the world's biological diversity are reviewed along with procedures in the natural products discovery process to estimate the potential that may still exist for the discovery of new bioactive compounds. For plants, it is estimated that the total number of species available is greater than has previously been recognized, and that there are perhaps close to 400,000 species of plants. Nevertheless, the percentage of species that have been effectively evaluated is limited by bias in collecting procedures, limits of bioassays, and procedures for bioassay, compound isolation, and structure elucidation. Furthermore, plants form only a small fraction of the world's total biological diversity, and lesser evident organisms, like insects and microorganisms, comprise both the greatest number of species and the greatest range of phylogenetic diversity (though some may present scale-up issues that will be difficult to solve). Several mechanisms are proposed to ensure greater efficiency of discovery procedures in the future, including methods to ensure collection of understudied taxa and methods to ensure that the full range of chemical diversity present in a given species is adequately surveyed.

Introduction

Plants have long been recognized as an important source for bioactive compounds. These are sometimes used directly as they occur in nature, as ingredients for pharmaceuticals, or as chemical structures that inspire synthetic or semisynthetic drugs (Farnsworth, 1984; Miller and Brewer, 1992; Grifo and Rosenthal, 1997; Newman et al., 2003). There have been numerous reviews of pharmaceutically active compounds from plants (e.g., Chadwick and Marsh, 1990), from which it is evident that drugs are derived both directly and indirectly from plants. Plants, as well as animals and microbial sources, yield bioactive compounds that are used as they are found in nature as ingredients in drugs, such as vincristine and vinblastine, the two anticancer drugs isolated from *Catharanthus roseus* (Farnsworth and Taylor, 1975). But more frequently, plants may yield compounds that are modified into semisynthetic drugs such as the cephalosporin antibiotics. In addition, the molecular structure of bioactive compounds may serve as scaffolds for the development of semisynthetic or completely synthetic drugs, as is the case for many modern antimalarials that are modified from the structure of quinine. Alternatively, they may help elucidate a biochemical reaction that facilitates design of an antagonist; it was not possible to envision the antihistamines until the natural product histamine was isolated from ergot.

The Natural Products Drug Discovery Process

Most modern efforts to discover new bioactive compounds from plants work with similar systems for the collection, processing, and evaluation of samples. Plants are collected and often divided into individual parts, such as leaves, stems, bark, roots, as these are assumed to differ in chemical composition. Generally, samples are dried, frozen, or otherwise processed to prevent them from degrading until such time as they can

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[2]Ruth Ann Bizoff helped assemble estimates of number of species, and Gerrit Davidse, Sandy Knapp, Nick Turland, and Fernando Zuloaga provided information on names and synonymy from various flora and monographic projects.

be extracted, usually at sites far from where they were collected. Samples are then ground into a fine powder and extracted, usually by extended extraction in an organic solvent, filtering, and evaporation of the extraction solvent, and sometimes followed by aqueous extraction (e.g., Cragg and Newman, 2001). Once solvents have been removed by evaporation, the resultant extracts generally form a thick, viscous material. These crude extracts may then be used directly in bioassays, or more frequently, they are fractionated by a wide variety of methods.

NATURE'S UNREALIZED POTENTIAL

Only a very limited number of plant species have been evaluated in modern bioassays, and published estimates of the percentage of plants that have been screened range upward from 0.5% (e.g., Balick, 1994). These are often misleading, as the majority of the plants have been evaluated only in a limited number of screens for a small number of therapeutic targets. The National Cancer Institute (NCI) has evaluated somewhere near 35,000 species of vascular plants in its 60-cell panel (Cragg and Newman, 2001; Newman, pers. comm.), but most other natural products programs that have evaluated large numbers of plants have often had bioassays cycle over relatively short periods, so that no single bioassay evaluates very large numbers of plants. Given that plants have yielded so many useful bioactive compounds yet only a small percentage of species have been evaluated against more than a limited number of targets, it would seem intuitive that there should still be significant numbers of medicinally promising compounds that remain undiscovered in plants. These useful bioactive compounds would include both potential drug ingredients and therapeutically promising chemical scaffolds that might provide blueprints for semisynthetic or wholly synthetic drugs.

Speculating about the potential for the discovery of new drugs from nature is a difficult exercise because of uncertainties estimating 1) the scope of biological diversity, 2) the scope of chemical diversity that exists in nature, and 3) the efficiency of the natural products discovery process and problems estimating how comprehensively plants have been evaluated against a nearly endless series of potential therapeutic targets. This paper attempts to quantify each of the various issues and make the multiple estimates that may be used to calculate how many potentially useful compounds remain to be discovered. The assumptions associated with each calculation will be considered, as well as the estimates of the low and high range. This is an effort to estimate the number of therapeutically useful novel bioactive compounds from plants that may exist undiscovered in nature, not an evaluation of the efficiency of natural products chemistry discovery programs. It is clear from the outset that only a small percentage of plants have been evaluated, that the sample of plants studied is a biased sample, that the full range of chemical diversity often has not been assayed, and that extracts have been evaluated against only a limited number of therapeutic targets. It is therefore easy to conclude, and generate numbers to support the notion, that only the surface has been scratched. What is certainly more interesting in this exercise is the more conservative estimate, not the greatest possible number, of the minimal number of compounds that may remain undiscovered.

HOW MANY DRUGS COME FROM PLANTS?

Plants have been an important source of the bioactive compounds used medicinally throughout human history (e.g., Chadwick and Marsh, 1990), in more than one way. Plants provide compounds that are directly used in pharmaceuticals, as they occur in nature, and they also provide compounds that are modified to produce semisynthetic drugs (Miller and Brewer, 1992; Grifo et al., 1997). But plants also have contributed to the development of many synthetic

mechanisms by providing chemical structures that allow medicinal chemists to synthesize compounds that would be difficult to imagine if the original pharmacophore from nature had not been discovered. Although a significant percentage of drugs have been reported to be derived from natural sources with naturally occurring active ingredients, semisynthetic active ingredients, or active ingredients altered or derived from natural structures (e.g., Miller and Brewer, 1992; Grifo et al., 1997), the list of active natural ingredients derived from plants is more limited in number. This less direct contribution of natural products to human health is therefore quite important, but it is much more complicated to estimate its potential contribution in the future. Therefore, this paper will focus on those compounds that may be used directly in the development of medicines.

Farnsworth (1990) identified 119 drugs from plant sources, and perhaps 16 additional new compounds had reached the market by 2002 (Newman et al., 2003). The estimate of 135 drugs from plant sources is conservative, because it only includes compounds derived directly from plants and used as they occur chemically in nature. It does not include semisynthetic products or synthetic medicines modeled on natural product pharmacophores. Given the long period of time that normally elapses from discovery through development, clinical evaluation, and approval, it can be assumed that these 135 drugs were discovered from plants evaluated prior to the major high-throughput screening efforts at most major pharmaceutical companies in the 1990s (ten Kate and Laird, 1999).

How Many Species of Plants Are There?

Estimates for the number of seed plants in the world have varied widely from the lower figures of 231,413 (Stebbins, 1974) and 260,000 (Thorne, 2002) to the much higher estimates of Govaerts (2001) and Bramwell (2002), both above 420,000. Stebbins and Thorne basically tried to count flowering plants, summing the available estimates for accepted species in each family one at a time, while Govaerts and Bramwell tried to estimate the percentage of published names that were synonyms. Stebbins (1974) followed chiefly the system of Cronquist (1968), with modifications according to the systems of Takhtajan (1966, 1970), Thorne (1968), and Hutchinson (1959), to create his own classification, which he then reviewed family by family, estimating the number of genera and species in each (Appendix, pp. 347–355) and summing them all to achieve his estimate. Thorne (2002) also estimated the total number of plant species by adding the number of species in genera as reported by Mabberley (1997) and supplemented with numbers from many recent revisions. The most recent edition of Mabberley (2008) reports a total of 272,370 species of seed plants.

Another approach that has been used to determine the number of described species of seed plants is to estimate the percentage of all published names that are synonyms. Govaerts (2001), Bramwell (2002), and Scotland and Wortley (2003) have made estimates using different means to estimate the percentage of published names that are synonyms, based on available revisions or floras. The process depends on estimating the percentage of synonymous names from checklists of particular families (e.g., Govaerts, 2001), regional floras (e.g., Bramwell, 2002) (which may be assumed to underestimate synonymy as they are often not inclusive of all synonyms and usually review only names used regionally), and a sample of monographs (e.g., Scotland and Wortley, 2003). The results from these exercises have produced estimates of synonymy that range from 58.4%–78% (Table 1), which in turn results in estimates of seed plant species ranging from 223,300 to 422,127. Prance et al. (2000) essentially adopt the basic count of accepted species from the range estimated by Stebbins (1974) and Thorne (2002) but then fo-

cus on a third element, which is the rate at which new species continue to be discovered and described. Thus, their estimate of 300,000 to 320,000 species of flowering plants really aims to guess how many species remain to be discovered and described.

From this discussion, it is evident that there are two components needed to estimate the total number of plants: the number of known species plus an estimate of the number of plant species that remain to be discovered, described, and named. The number of known species can be estimated either by counting accepted names or from the total number of species names that have been published; this number is available from the International Plant Names Index (IPNI) minus the names estimated to be synonyms. Because many species have been described and named more than once, the number of published names always exceeds the number of species actually recognized in any plant group, so it is essential to estimate the percentage of synonymous names in order to calculate the total number of accepted names. This is difficult to estimate because of unevenness of taxonomic knowledge of groups and different rates of synonymy from group to group. Thus, any estimate of the total number of plants must determine the percentage of published names that represent unique, accepted species plus an estimate of the number of species that remain to be discovered, named, and described.

Several other estimates were gathered as part of the present review to determine if they were consistent with previous estimates. A sample of the revisions published in *Systematic Botany Monographs* was reviewed (Table 2). Only revisions of seed plants were included. Thus, revisions of ferns and fungi were excluded, and revisions of geographic subsets of taxa (other than New World or Old World) were excluded as they often underestimate synonymy. The results of these reviews show that the sample of revisions consulted in *Systematic Botany Monographs* produced an average synonymy rate of 44%, a rate much lower than previous estimates, which

Authors	Estimated Percent Synonymy	Total Estimated Seed Plant Species
Scotland and Wortley (2003)	78.0%	223,300
Stebbins (1974)	77.2%*	231,413
Mabberley (1997)	75.4%*	250,140
Mabberley (2008)	73.2%*	272,370
Thorne (2002)	74.3%*	260,000
Prance et al. (2000)	68.4%–70.4%	300,000–320,000
Bramwell (2002)	58.4%	421,968
Govaerts (2001)	58.4%	422,127
This paper:		
New World Grasses	78.1%	222,285
Solanum	76.7%	236,495
Flora of China	72.8%	276,080
Southern Cone	70.2%	302,470
Syst. Bot. Monographs	44.0%	446,600

Table 1. Estimates from recent publications of rates of synonymy and the total number of seed plant species. Data for *Catalogue of New World Grasses* from: http://mobot.mobot.org/W3T/Search/nwgc.html; *Solanum* (Knapp, pers. comm.); *Flora of China* (Turland, pers. comm.); *Catálogo de las Plantas Vasculares del Cono Sur* (Zuloaga, pers. comm.).*Synonymy rates calculated retrospectively.

Taxon	Family	Total No. of Binomials	Accepted	Synonyms	Percent Synonymy	Reference
Lygodesmia	Asteraceae	8	8	0	0%	Tomb, 1980
Lomatium	Apiaceae	21	13	8	38%	Schlessman, 1984
Lilaeopsis	Apiaceae	24	13	11	46%	Affolter, 1985
Cordylanthus	Scrophulariaceae	22	13	9	41%	Chuang & Heckard, 1986
Agastache	Lamiaceae	20	14	6	30%	Sanders, 1987
Desmanthus	Fabaceae	51	24	27	53%	Luckow, 1993
Poitea	Fabaceae	21	12	9	43%	Lavin, 1993
Pappobolus	Asteraceae	41	38	3	7%	Panero, 1992
Clermontia	Campanulaceae	54	22	32	59%	Lammers, 1991
Muhlenbergia	Poaceae	61	29	32	52%	Peterson & Annable, 1991
Simsia	Asteraceae	52	18	34	65%	Spooner, 1990
Chusquea	Poaceae	35	31	4	11%	Clark, 1989
	Eremolepidaceae	22	12	10	45%	Kuijt, 1988a
Tristerix	Loranthaceae	25	11	14	56%	Kuijt, 1988b
Nymphaea	Nymphaeaceae	23	14	9	39%	Wiersema, 1987
Leochilus	Orchidaceae	20	9	11	55%	Chase, 1986
Tetramerium	Acanthaceae	51	28	23	45%	Daniel, 1986
Acmella	Asteraceae	83	30	53	64%	Jansen, 1985
Maurandyinae	Scrophulariaceae	22	20	2	9%	Elisens, 1985
Cobaea	Polemoniaceae	25	18	7	28%	Prather, 1999
Gasteranthus	Gesneriaceae	51	35	16	31%	Skog & Kvist, 2000
Totals		732	412	320	44%	

Table 2. Estimates of synonymy for recently monographed plant taxa published in *Systematic Botany Monographs*. The average rate of synonymy is 44%.

would mean that there are 568,000 accepted names already published, a difficult figure to believe. It is not clear why these revisions present a rate of synonymy that is much lower than the estimates obtained by Govaerts (2001) or Scotland and Wortley (2003). It may simply be that the taxa revised in these monographs were selected because of the feeling that they needed work, meaning that large numbers of species needed to be described, hence the low synonymy.

Data were also reviewed from several major regional flora projects. Fernando Zuloaga (pers. comm.) reports that the flora of the Southern Cone (Argentina, Chile, Uruguay, Paraguay) includes 67,000 names for about 20,000 species, a rate of about 70.2% synonymy. The *Flora of China* includes 115,000 names for 31,300 accepted species (Turland, pers. comm.), a synonymy rate of 72.8%. These two rates of synonymy would result in estimates of the world's plants of 276,080 and 302,470, much more in line with most other estimates.

In the review of the taxonomy of *Solanum* currently under way (Knapp, pers. comm.), 76.7% of the names that have been reviewed have been synonyms. In the *Catalogue of New World Grasses*, 28,699 names are included, but only 6,296 are accepted and 78.1% are considered synonyms (Judziewicz et al., 2000; Peterson et al., 2001; Soreng et al., 2003; Zuloaga et al., 2003; and online at http://mobot.mobot.org/W3T/Search/nwgc.html).

The final component needed is an estimate of the number of species that remain to be dis-

Year	No. of New Species Described
1989	2,752
1990	2,653
1991	2,220
1992	1,705
1993	2,049
1994	2,126
1995	2,413
1996	2,409
1997	2,770
2004	1,898
2005	1,744

Table 3. Rate of description of new seed plant species from 1989 to 1997 (data from *Index Kewensis*; see Prance et al., 2000) and 2004 and 2005 (data from IPNI).

covered and described. Prance et al. (2000) reviewed several sources to make an estimate including information from *Index Kewensis* dating from 1989–1997, and these numbers, as well as the number of new species described in 2004 and 2005, available from IPNI, are summarized in Table 3. During this period, new species were described at an average of 2,249 per year. There is no evidence that the rate of discovery and description of new species is declining, nor will it decline in the near future. This number is thus more probably a measure of the descriptive capacity of the taxonomic community.

Clearly, we are a long way from knowing the world's flora, and parts of South America, Madagascar, and tropical Asia continue to yield tremendous numbers of new species. Furthermore, there is no evidence that they will be completely explored any time in the near future (Prance et al., 2000). Thomas (1999) reviewed 76 monographs of Neotropical plant groups from *Flora Neotropica* monographs and found that 29% of the species included were newly described. A review of revisions of Malagasy plant genera published in *Adansonia* (Table 4) shows that since 2000, there have been 274 species treated, 105 (or 38%) of which were new to science. Madagascar is probably exceptional, even within the tropics, and the percentage of species that remain to be described from continental Africa would probably be lower. On the other hand, tropical Asia might present similar figures, so perhaps one third of tropical species remain to be named. There are probably few temperate species that remain to be described, but Prance (1977) and Raven (1988) estimated two thirds of the total number of plants, or 154,275 to 213,827 species, are tropical. If one third remain to be described, that would suggest that there are still 51,425 to 71,256 additional species. Prance et al. (2000) and the data in Table 3 support the idea that the taxonomic community describes roughly 2,000 new species per year, and this seems unlikely to diminish in the near future. Although it is difficult to project when in the future this rate might begin to slow, it does not seem to be inconsistent with the estimate of one third of tropical plants.

Deforestation in the tropical regions, where most of the species remaining to be discovered exist, continues at an alarming rate. At some point in the future, the impact of deforestation will intersect with continued exploration and collection, and the last remaining forests will have known species compositions. Schatz et al. (2000) predicted that forests outside of the protected area system in Madagascar would essentially be completely deforested in the next 20 to 30 years. It is not clear when this intersection of deforestation and exploration will occur, but since the discovery of new plant species takes place both in the wild and in herbarium collections, it seems unlikely that the rate of description of new plant species will decline in less than 50 years. Even conservatively assuming that half of the species still to be described will ultimately be placed in synonymy, there would still be 25,000 to 35,000 species beyond those already accepted.

Given the range of estimates for synonymy reported in the literature (Table 1) and calculated for *Systematic Botany Monographs* and *Flora*

Genus	Family	No. of Species	No. of New Species	Reference
Apodytes	Icacinaceae	4	1	Labat et al., 2006
Aspidostemon	Lauraceae	28	18	van der Werff, 2006
Bathiorhamnus	Rhamnaceae	7	2	Callmander et al., 2008
Beilschmiedia	Lauraceae	9	2	van der Werff, 2003
Brexia	Celastraceae	9	3	Schatz & Lowry, 2004
Bulbophyllum p.p.	Orchidaceae	11	2	Bosser, 2000
Buxus	Buxaceae	9	7	Schatz & Lowry, 2002
Cryptostegia	Apocynaceae	2	0	Klackenberg, 2001
Cynoglossum	Boraginaceae	6	3	Miller, 2005
Disperis	Orchidaceae	22	6	LaCroix et al., 2002
Drypetes	Euphorbiaceae	11	1	McPherson, 2000
Ehretia	Boraginaceae	7	5	Miller, 2002
Hilsenbergia	Boraginaceae	13	12	Miller, 2003
Leptolaena p.p.	Sarcolaenaceae	8	5	Schatz et al., 2001
Micronychia	Anacardiaceae	5	1	Randrianasolo, 2000
Octolepis	Thymelaeaceae	5	3	Rogers, 2005
Olax	Olacaceae	8	3	Rogers et al., 2006
Operculicarya	Anacardiaceae	8	2	Randrianasolo & Lowry, 2006
Pandanus	Pandanaceae	6	1	Laivao et al., 2007
Phanerodiscus	Olacaceae	3	1	Malécot et al., 2003
Pseudophyllanthus	Euphorbiaceae	3	2	Hoffman, 2000
Pyrenacantha	Icacinaceae	9	5	Labat et al., 2006
Rhodolaena	Sarcolaenaceae	7	3	Schatz et al., 2000
Stephanodaphne	Thymelaeaceae	9	3	Rogers, 2004
Talinella	Portulacaceae	12	7	Applequist, 2005
Xyloolaena	Sarcolaenaceae	5	2	Lowry et al., 2002
3 genera (includes *Eremolaena*, *Pentachlaena*, and *Perrierodendron*)	Sarcolaenaceae	10	5	Lowry et al., 2000
Total		274	105 (38%)	

Table 4. Recently published revisions of plant genera from Madagascar with total number of plant species treated and the number that are newly described.

Neotropica, it is possible to calculate a range for the number of known, accepted species. When an estimate for the number of species that will be described in the future is added, it should be possible to estimate a range for the number of seed plant species that exist. There seems to be reasonable congruence between estimates that attempt to count plants (Stebbins, 1974; Mabberley, 1997, 2008; Thorne, 2002) and either efforts to estimate percent synonymy from large floristic data sets (Wu and Raven, 1995–present; Zuloaga, pers. comm.) or estimates from comprehensive checklists from working groups of specialists (Judziewicz et al., 2000; Knapp, pers. comm.; Peterson et al., 2001; Soreng et al., 2003; Zuloaga et al., 2003), all of which range between 231,413 and 302,470. The estimates from samples of revisions (Scotland and Wortley, 2003 and the review of *Systematic Botany Monographs* reported here) both fall outside of this range and will not be further considered. In addition, the estimates of Bramwell (2002) (which add highly

speculative numbers from floristic data) and Govaerts (2001) (which rely on world checklist data) are both outside of all other congruent estimates. For the purposes of this paper, the known number of seed plants is considered to fall between 272,370 (the most recent count of Mabberley in 2008) and 302,474 (the highest reasonable estimate from estimated synonymy). With 25,000 to 35,000 species remaining to be discovered and described, the total estimate for seed plants would then range between 297,370 and 337,470. Rounding these numbers, the lower and upper estimates for the total number of seed plants will be considered to be 300,000 to 340,000.

How Many Plants Have Been Screened and How Effectively?

While there are numerous publications that claim that some specific percentage of plants have been screened in drug discovery programs (e.g., 5%–10%, Svendsen and Scheffer, 1982), and generally these range from a few percent to slightly more than 10%, it does not take much investigation to realize that these estimates are not accurately informative for a number of reasons. Although the most ambitious screening programs, like the NCI's Natural Products Program (Cragg and Newman, 2001), may have screened more than 100,000 samples, they probably have still screened fewer than 35,000 species (Newman, pers. comm.). The larger number of samples includes multiple plant parts per collection, as well as repetitive gatherings of the same species, so the NCI has sampled only about 8%–16% of the world's plant species, depending on the estimate of the world's flora.

The taxonomic diversity of plants, clearly correlated with chemical diversity, has not been evenly sampled for several reasons. Plant diversity is not evenly distributed geographically in terms of either abundance or distribution. The results of a large series of ecological transects conducted by Alwyn Gentry and his colleagues (Phillips and Miller, 2002) demonstrate that forests range in diversity from having nine to 275 species of woody plants 2.5 cm or more in diameter at breast height per tenth of a hectare. One of the consequences of the uneven distribution of plant diversity is that efforts to sample plant diversity in bioprospecting programs generally results in biased samples (Miller and Gereau, 2000; Miller et al., 2005). In addition, small plants that are difficult to gather in adequate amounts, and plants with limited distributions, or those that, for other reasons, are unlikely to be encountered, are underrepresented in screening programs.

The ultimate result is that, because some geographic areas remain uncollected, combined with a bias toward collecting plants that are easier to gather in quantity, some taxa remain completely unstudied.

The majority of screening programs evaluate extracts against a single or limited number of therapeutic targets. Newman et al. (2003) recognize 55 therapeutic areas where four or more new chemical entities were introduced into the drug market between 1981 and 2002, and most of these areas include a vast array of diseases; anticancer is one of the therapeutic targets, yet NCI recognizes that the term *cancer* encompasses more than 300 distinct diseases. In addition, Newman et al. (2003) recognize more than another 100 therapeutic areas where one to three new chemical entities were introduced to the drug trade during the same period, and their report includes only therapeutic areas where new drugs were introduced into the market during the 21 years of their survey; there are clearly many disease areas that did not have newly developed drugs. It should be immediately apparent that many of their categories (e.g., antibacterial, anticancer, antifungal, antiparasitic, antiviral) consist of nearly endless lists of individual diseases, and thus the list of therapeutic targets against which a given extract might be evaluated is also nearly endless. There are probably no plant species that can be con-

sidered to have been evaluated against all possible therapeutic targets.

Finally, recognizing how ineffectively the natural world has been evaluated in the search for new drugs, Suffness and Douros (1982) wrote "it quickly becomes clear that in order to be detected an active compound would have to be either quite abundant or extremely potent and that it is highly likely that in vitro screening alone is going to miss compounds which may be quite active but are not potent enough or not concentrated enough in a crude extract for detection." Beyond the question of how many species have been extracted and evaluated, one must ask how effectively the extracts that have been delivered have been evaluated. Plant extracts are composed of hundreds of compounds (Eldridge et al., 2002), yet the history of natural products research is largely of screening whole plant extracts, extracts that have had confounding compounds, such as tannins, removed, or extracts following limited fractionation. Recently, several publications have described methods to partition extracts into smaller fractions, or libraries, so that compounds can be surveyed in bioassay systems and neither lost because of low concentration, nor have their activity masked by a group of other compounds, nor have their activity interfered with because of competitive compounds (Cremin and Zeng, 2002; Eldridge et al., 2002).

The only conclusion that can be drawn from these observations is that while a modest percentage of plants have been screened in one program or another, only a very few have been evaluated in a manner that assays their full range of chemical diversity and does so against a broad enough range of therapeutic targets. Perhaps the only species that can be assumed to have had their chemistry fully evaluated are those that have proven of interest for one reason or another and have subsequently had their chemical composition fully characterized. This list could include food plants, other species of commercial importance for a variety of reasons, and the plant species (and some of their relatives) that have yielded important bioactive compounds. Yet at the same time, numerous plant samples have been screened, compounds have been discovered and developed, and new medicines have made it to the market. It is clear that many species have not been evaluated, and for those that have been evaluated, it has mostly been against limited numbers of targets; only a few have had their full range of chemistry studied.

For the purposes of the calculations that are the primary goal of this paper, it is necessary to make several assumptions about the number of species that have been evaluated. The important question to answer to complete these calculations is how many plants were evaluated to discover the 135 drug ingredients. Given the discussion above, it should be clear that the only species that have been thoroughly evaluated are those that have had either their full range of chemical composition carefully assayed against a wide array of therapeutic targets or have had their full chemical composition characterized for other reasons. The number of species that meet either of these criteria would be small and probably limited to major food plants, plants that yield oils, or plants known to have interesting medicinal characteristics. Although there is documentation that humans consume about 10,000 food plants (Tanaka, 1976), fewer than 100 supply the vast majority of the world's food supply and most of the other species remain poorly known in terms of their chemistry. There are also medicinal plants and their relatives that have been very thoroughly studied (e.g., *Catharanthus* G. Don spp., Farnsworth and Taylor, 1975). Although it is nearly impossible to estimate accurately how many plants are thoroughly known chemically, it may be only a few hundred. Two thousand is used here as a conservative estimate of the number of plants that are well known.

The other number that it is necessary to estimate is an upper limit of the number of plants that were studied, even if not thoroughly, to discover the 135 active drug ingredients. Given the long time period between discovery through development and clinical evaluation to approval, the discovery of all or very nearly all of the 135 active ingredients must predate the modern high-throughput discovery efforts made at most major pharmaceutical companies during the 1990s (ten Kate and Laird, 1999). Probably the most ambitious program to evaluate plants has been that of NCI. The early phase (from 1956 to 1974) evaluated 78,882 extracts, which probably represent 25,000 to 40,000 plant species (Shepartz, 1976). The more recent phase of the program (1986–present) has evaluated about 35,000 species (D. Newman, pers. comm.). The discovery of the vast majority, and perhaps all, of the 135 compounds would predate 1986, so the high estimate of 40,000 samples from the first phase of NCI research will be used as an upper limit on the number of plants that were screened by the NCI. Even though these samples were only evaluated in assays for cancer, and most were evaluated only as whole organic or aqueous extracts (although some compounds were discovered), the plants studied may still have useful compounds that remain to be found. Although other species were evaluated in other research programs, given that all or nearly all of the 135 compounds were discovered prior to the introduction of modern high-throughput methods, no one has evaluated the large number of species more typical of programs since the 1980s. Most of the efforts to screen plants in programs other than the early NCI effort would most likely have had great overlap in the species studies, and it seems unlikely that the sum of these other research efforts would have increased the number of plants evaluated by more than another 50%, so 60,000 is considered here to be the upper limit of the number of plants evaluated to discover the 135 known drug ingredients.

DISCUSSION

The Calculations and Assumptions

The estimates of known species of seed plants range from the lower count from Mabberley (2008) of 272,370. If there are approximately 1,015,000 published names of seed plants (Govaerts, 2001), with the reasonable estimates for rates of synonymy (Table 1) ranging as low as 70.2%, the upper estimate for the total number of seed plants would be 302,470.

Assumption One

Only a percentage of published names represent accepted species, so it is assumed that if rates of synonymy are estimated, these estimates can divide published names into accepted names and synonyms and allow an estimate of known species. The range of estimates for synonymy vary tremendously (Table 1), and lower estimated rates of synonymy result in higher estimates for the number of known plant species. As noted before, the estimates from samples of monographs vary widely and seem misleading—about half of the plants in Scotland and Wortley's (2003) estimate are conifers, primarily from temperate regions, so their estimate for synonymy is high and results in an estimate for total number of species that is low. On the other hand, the revisions from *Systematic Botany Monographs* reviewed here (Table 2) are primarily the first comprehensive modern reviews; many of the taxa are tropical, and descriptions are, to a greater degree, of new species, with fewer names placed in synonymy. These extremes are assumed to be unrepresentative and are therefore excluded. This assumption is carried into the next calculation, as it is assumed that only a percentage of the new names published each year will remain as accepted names. For purposes of these calculations, the estimates that are extreme and based on uneven samples of monographs or checklists (Govaerts,

2001; Scotland and Wortley, 2003; Table 2) and the estimate of Bramwell (2002) are excluded.

The present rate of describing ca. 2,000 seed plant species per year is probably more reflective of the descriptive capacity of the taxonomic community, and the estimate that one third of tropical species remains unnamed is not inconsistent with this rate and seems reasonable. This would result in an additional 50,000 to 70,000 names for new seed plant species. If half remain accepted, there would be 25,000 to 35,000 species of plants awaiting description. Adding both the low and high estimates for new species that will be described to the numbers calculated above for known seed plant species pushes the range for the estimates of how many actual seed plant species exist from about 300,000 to 340,000. The lower range is not too far beyond efforts to count known plant species (Table 1), and the upper is not far beyond the speculations of Prance and colleagues (2000).

Assumption Two

It is difficult to guess how long the current average rate of publication of new plant names (about 2,000 per year) will continue. There is no evidence that the rate of description of new species is slowing significantly, and the present number is probably more reflective of the capacity of the taxonomic community to name new species. The more difficult question is this: how long can this continue? Eventually, exploration should begin to yield novelties at a decreasing rate. But perhaps more significant is the problem that deforestation and degradation of natural habitats continue at an enormous pace. Some have predicted that in less than 50 years, the only remaining major forests in the world will be in protected areas (Schatz et al., 2000). Because of this uncertainty, the estimate that about one third of tropical species remain to be described is adopted for these calculations, and about one half of these will ultimately be assigned to synonymy. The plant species to be described therefore only comprise about 10% or less of the total estimate for existing seed plant species, so this does not have a great effect on the total.

For purposes of the calculations here, the number of plant species that have been evaluated in pharmaceutical discovery programs is considered to range from 2,000 to 80,000. The lower number is a generous estimate of those plant species that have either been evaluated against a broad array of therapeutic targets or are well characterized chemically. The upper estimate of 80,000 would be a conservative estimate (50% beyond the NCI program) of the number of plants that were studied in all pharmaceutical programs, recognizing that the vast majority were evaluated against only one or a limited number of targets and may still contain undiscovered bioactive compounds.

Assumption Three

It is difficult to accurately assess how many plant species have been evaluated in one or more bioassay systems. It seems logical that few have been screened against more than a minimal number of potential therapeutic targets, but as details from most of the commercial screening programs are not publicly disclosed, it is difficult to make accurate guesses.

The calculations for the number of drug ingredients that remain to be discovered are based on the 135 known natural product drug ingredients reported by Farnsworth (1990) and two estimates for the number of plant species that were screened to discover them. The number of plant drug ingredients that remain to be discovered thus can be calculated as:

$$\frac{135}{\text{\# of plant species screened}} = \frac{\text{\# of drug ingredients to be discovered}}{\text{Total \# of seed plant species} - \text{\# of plants screened}}$$

By substituting the two estimates for numbers of plants that have been screened and the two estimates for the total number of seed plant species, it is possible to calculate the number of new bioactive compounds that remain to be discovered.

Assumption Four

The above calculations are based on the assumption that each subsequent plant species evaluated has the same probability of yielding a new drug ingredient as those that have previously been screened. This assumption is almost certainly not true. The likelihood that evaluation of a previously unstudied species will yield a novel bioactive compound decreases with the number of plants evaluated. Many secondary metabolites are of common occurrence in plants, so as additional plant species are added, the likelihood is that a smaller percentage of compounds in their extracts will be new to any given bioassay decreases as the number of plants studied increases. It is also true that not all new plants introduced into a given assay system have an equal probability of yielding new discoveries. Adding a new member of a large plant genus, from which many congeners have previously been evaluated, will have a much smaller chance of yielding novel bioactives than will a species from a family yet to be evaluated in the screening system. In fact, the "novelty" of the taxonomic identity of any given species introduced into a pool of species to be screened may have a dramatic effect on its chances to yield different chemistry. Perdue (1976) went so far as to suggest that most species of the same genus were not likely to differ significantly in their secondary chemistry, though there are certainly many well-known examples contrary to this assumption (e.g., only two of the ca. 180 species of *Erythroxylum* P. Browne are known to yield cocaine [Mabberley, 1997]). For these reasons, what is difficult about this assumption is getting a measure on how significantly it overestimates the likelihood that screening additional new species will continue to yield new compounds at the same rate. Because of this, it is easy to expect that the estimated numbers of new drug ingredients that remain to be discovered in Table 5 are high; it is probably nearly impossible to know by how much.

CONCLUSIONS

Despite the limitations of the assumptions outlined above, it is clear that there are probably significantly more plant species than have previously been thought, probably as many as the 300,000 to 320,000 that Prance et al. (2000) proposed, or perhaps even slightly more. It is also clear that only a modest percentage of these species has been evaluated in any screening efforts, and only a minute percentage could be considered well known in terms of their chemical composition. Given that only a few plant species have been evaluated in multiple screens and many of them have been evaluated as whole extracts in a manner that may miss all but the compounds present at high concentrations, the plant world still seems very understudied. While difficult to estimate with a high confidence level, it is clear that there are many potentially useful bioactive compounds that remain to be discovered.

The predicted numbers of possible undiscovered drug ingredients from Table 5 provide estimates that bracket the most probable range of undiscovered compounds. The lower estimates, resulting from the assumption that 60,000 plants have been evaluated, indicate that screening even crude extracts of the unstudied species against a modest range of therapeutic targets would likely discover more potential drug ingredients than are known to date. At the other extreme, assuming that only 2,000 species are well known chemically, and assuming that every species of plant would be well characterized chemically and have each secondary metabolite evaluated in a large number of pharmaceutical screens, supports

Estimated No. of Seed Plant Species	Total No. of Seed Plant Species Screened	
	2,000	60,000
300,000	20,115	540
340,000	22,815	630

Table 5. The number of new drug active ingredients that remain to be discovered assuming different numbers of seed plant species and that different percentages of plant species have been evaluated to yield the 135 active drug ingredients currently known.

the prediction that we have barely scratched the surface and that the vast number of medicinally useful compounds from plants remain undiscovered. The most realistic estimates are probably somewhere between these numbers, but one important factor needs to be considered in their interpretation: the chemical redundancy that exists between species, particularly those that are closely related. Perdue (1976) suggested that species within genera were most likely chemically redundant, and with about 13,000 known plant genera (Mabberley, 2008) that would reduce the predictions in Table 5 to about 4% or 5% of their values. However, this adjustment for chemical redundancy is almost certainly too extreme. There are numerous examples of chemical variability within genera, and most researchers easily accept that there are still useful compounds to be discovered from plants that have had some previous evaluation.

This article attempts to provide an estimate of undiscovered useful bioactive compounds from one group of organisms, plants. While plants are tremendously diverse, many remain undiscovered and only a small percentage have been evaluated in bioassays; the vast majority of biodiversity, and hence chemical diversity, lies in other groups of organisms that may be even more poorly studied than plants. Wheeler (1990) showed that the vast majority of biological diversity, hence chemical diversity, resides in lesser evident organisms, such as insects, fungi, and microbes. Thus, the estimate of new bioactive compounds that remain to be discovered from plants is only a portion of the medically useful chemical diversity that exists in nature.

Beyond the predictions that result from the calculations, which predict that a significant percentage of potential drug ingredients from plants remain undiscovered, a number of recommendations for natural products discovery efforts in the future result from the calculations and discussion above. First, it will be important to continue to develop new bioassays and increase the breadth of therapeutic targets against which samples can be evaluated. Second, it will be important to continue to collect plants and build taxonomically diverse libraries of samples. Third, the libraries of plant materials should be extensively fractionated prior to bioassay to reduce interference between compounds, masking of activity, and to allow adjustment of concentration so that all compounds evaluated are present in sufficient concentration to register in bioassay systems. If, in the future, plants are studied in greater numbers, against a more comprehensive array of therapeutic targets and in a manner that fully evaluates the complete range of secondary compounds that they possess, it is clear that there are many more potential drug ingredients in plants that remain to be discovered than have been discovered and used in the past.

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Natural Products as Sources for Drug Development: The Case of Paclitaxel (Taxol)[1,2]

David G. I. Kingston[3]

The approach to new drugs through natural products has proved to be the single most successful strategy for the discovery of new drugs. This article reviews some of the past successes of the natural products approach, with an emphasis on plant-derived natural products with anticancer activity. The most successful plant-derived anticancer agent is paclitaxel (Taxol, Bristol-Myers Squibb, New York, NY), and the chemistry of paclitaxel and the paclitaxel-tubulin interaction is then described, with an emphasis on studies that led to the discovery and experimental proof of the T-taxol conformation as the tubulin-binding conformation of paclitaxel. The implications of this conformation for future drug development are also briefly covered.

Introduction

Plant-derived Natural Products as Anticancer Agents

Natural products, or "nature's combinatorial library" have been the source of many drugs, especially anticancer drugs. Thus half of all prescriptions dispensed in the U.S. contain substances of natural origin, one quarter of all prescriptions contain a plant-derived active principle, and over $8 billion of U.S. prescription drugs in 1980 were estimated to be plant based (Principe, 1985). A recent authoritative review concluded that 61% of all the new drugs introduced worldwide during 1981–2002 can be traced to or were inspired by natural products (Newman, Cragg, and Snader, 2003). The reasons for the importance of plant natural products as drug leads and drug substances are varied, but they include as a minimum the fact that many plants need to protect themselves against predation by the production of various defensive substances that make them inedible or toxic. It has in fact been argued that "all natural products have evolved under the pressure of natural selection to bind to specific receptors" (Williams et al., 1989), and if this view is correct it is not surprising that some subset of natural products will be found to have physiological effects in humans. In addition to this consideration, natural products are usually chiral compounds and are often structurally complex, so they are not accessible by the normal methods of synthetic medicinal chemistry. This point is made very clearly by the work of Feher and Schmidt (2003), who show that natural products in general are more "drug-like" than the products of synthetic combinatorial chemistry.

Various categories of plant-derived compounds have become important pharmaceutical products. These include: alkaloids such as atropine, camptothecin, codeine, colchicine, emetine, pilocar-

[1]The work from the author's laboratory was carried out by a large group of talented and hardworking colleagues and collaborators. The tubulin and REDOR studies were done in collaboration with Dr. Susan Bane (SUNY Binghamton) and Dr. Jacob Schaefer (Washington University, St. Louis), and the modeling work was carried out by Dr. Jim Snyder (Emory University), and I am enormously grateful to them and their colleagues for these exciting results. Recent work at Virginia Polytechnic Institute and State University was done by Dr. T. Ganesh, Chao Yang, and Shoubin Tang; earlier work was carried out by colleagues whose names are given in the references cited. Financial support was provided by the National Cancer Institute from grants CA55731 and CA69571, and I am most grateful for this crucial component of our work.

[2]The name Taxol is a registered trademark of Bristol Myers Squibb for their formulation of the compound shown in Figure 5A. The chemical name taxol was, however, originally given to the chemical compound shown in Figure 5A by its discoverers. In view of the historical nature of this review, the word taxol is retained for the chemical substance shown in Figure 5A. No infringement of the BMS trademark is implied.

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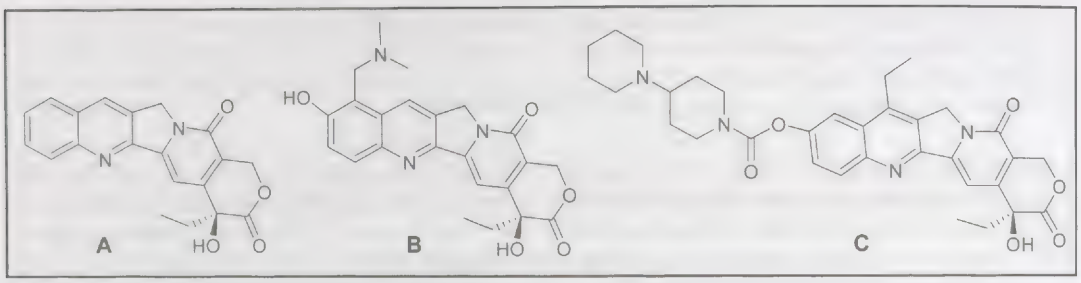

Figure 1. A: camptothecin; B: topotecan; C: irinotecan.

pine, reserpine, quinine, and vinblastine; terpenes such as artemisinin, diosgenin, and taxol; and phenolics such as podophyllotoxin and combretastatin (Raskin et al., 2002). The following section gives some examples of plant-derived clinically used anticancer agents.

Camptothecin

Camptothecin (Figure 1A) was isolated from *Camptotheca acuminata* Decne. in 1966 by Wall et al. (1966). It had potent anticancer activity in preliminary in vitro and animal assays, but its development was hampered by its extreme insolubility in water and the associated difficulties in formulation, and it was dropped from development. Interest in it as an anticancer agent was revived when it was discovered that it operated by the then-new mechanism of inhibition of topoisomerase I (Hsiang et al., 1985), and the two water-soluble derivatives topotecan (Hycamtin; SmithKline Beecham, now Glaxo SmithKline, London, U.K.; Figure 1B) and irinotecan (Camptosar; Pharmacia and Upjohn, now Pfizer, New York, NY; Figure 1C) were developed. These compounds are used clinically for treatment of advanced ovarian cancer (topotecan) and advanced colorectal cancer (irinotecan), as well as other cancers (Rahier, Thomas, and Hecht, 2005).

Combretastatin

The first significant compound of the combretastatin class to be isolated was (-)-combretastatin (Figure 2A) from *Combretum caffrum* Kuntze (Pettit et al., 1982); it is worth recording that Gordon Cragg was a coauthor on this important publication. Subsequent studies resulted in the isolation of many additional combretastatins, including combretastatins A1 (Figure 2B) and A2 (Figure 2C), which were two of the most active members of this class, with potent activity as inhibitors of tubulin assembly. Further development by the Pettit group led to the discovery of combretastatin A4 phosphate (Figure 2D) as a promising drug candidate, and it was subsequently found to be an important member of a new class of compounds known as tumor-specific vascular targeting agents. It received orphan drug approval by the FDA in 2003 for the thyroid cancer group, and the FDA has approved it

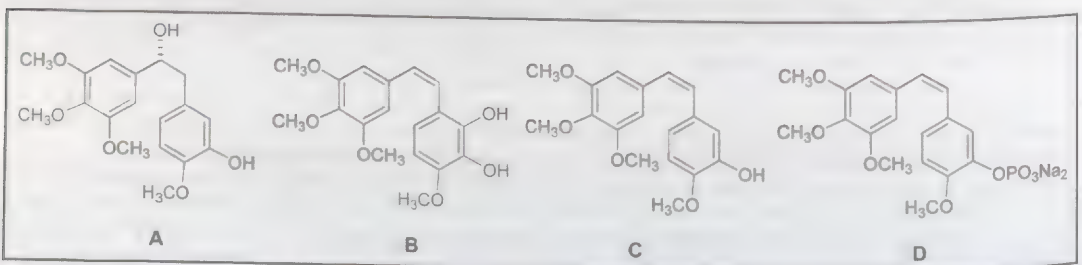

Figure 2. A: (-)-combretastatin; B: combretastatin A1; C: combretastatin A2; D: combretastatin A4 phosphate.

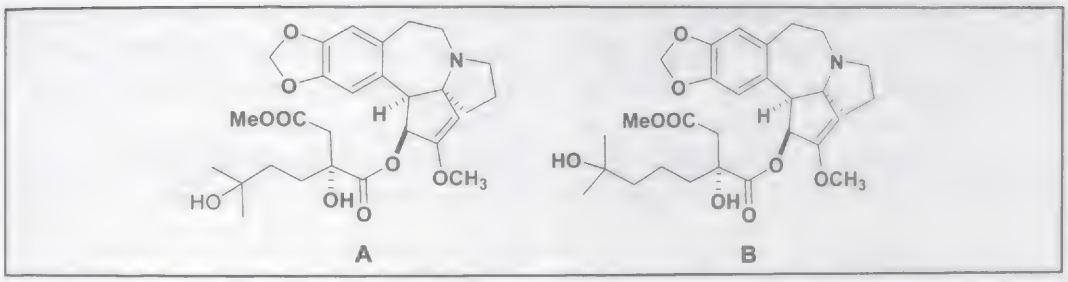

Figure 3. A: harringtonine; B: homoharringtonine.

for a "fast-track" phase II clinical trial against anaplastic thyroid cancer (Pinney et al., 2005).

Homoharringtonine

Harringtonine (Figure 3A) and its homolog homoharringtonine were isolated from the Chinese evergreen tree *Cephalotaxus harringtonia* (Knight ex Forbes) K. Koch var. *harringtonia*, and their structures were determined by Powell et al. (1970). These compounds and numerous analogs have been investigated as anticancer agents. They function as protein synthesis inhibitors, and thus differ from the other natural products described in this section that are inhibitors of tubulin assembly or disassembly, or of one of the topoisomerases. Although homoharringtonine (Figure 3B) has found clinical use in the People's Republic of China, neither it nor harringtonine have yet been approved for clinical use in the U.S. Their major activity is as antileukemic agents, and homoharringtonine has been shown to be effective against chronic myeloid leukemia. Further details can be found in Itokawa, Wang, and Lee (2005).

Podophyllotoxins

Members of the genus *Podophyllum* L. (Podophyllaceae) have been used for centuries as medicinal agents, and so the constituents of plants of this genus were investigated for their medicinal properties from as early as the 1940s. Podophyllotoxin, the major constituent of the rhizome of the American May Apple, *P. peltatum* L., was shown as early as 1947 to inhibit formation of the mitotic spindle (Sullivan and Weshler, 1947). Subsequent work by Hartwell and Schrecker (1951) led to the final structure of podophyllotoxin (Figure 4A). Parenthetically, it can be noted that Jonathan Hartwell's interest in this compound, and hence in the potential of natural products in general as anticancer agents, was key to

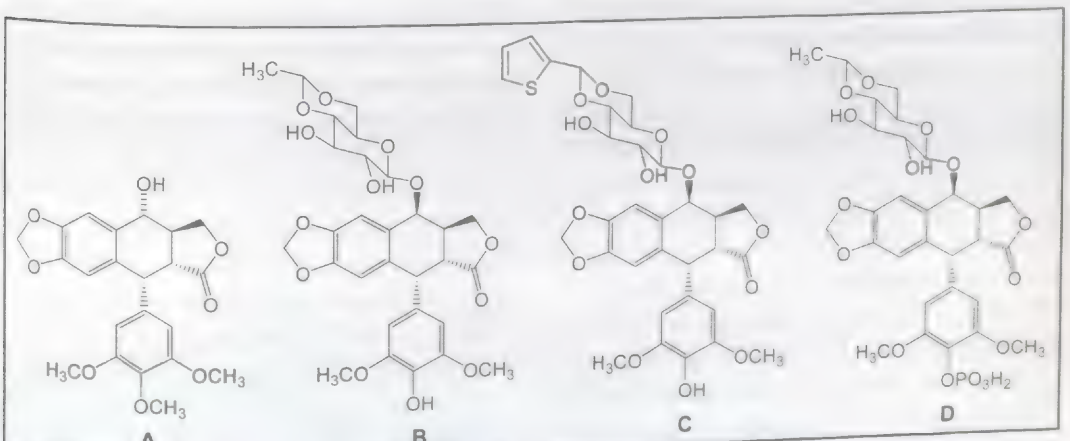

Figure 4. A: podophyllotoxin; B: etoposide; C: teniposide; D: Etopophos.

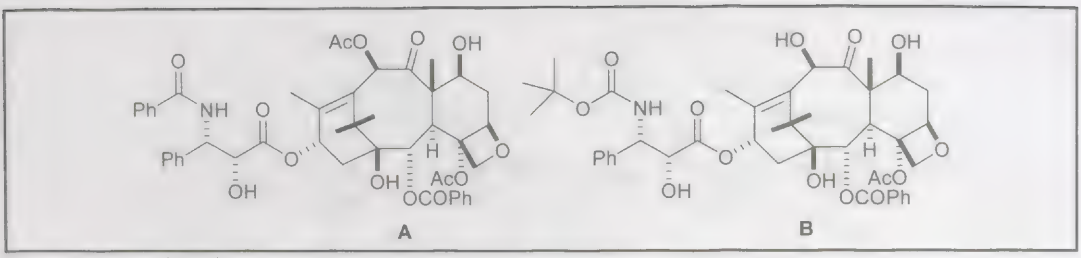

Figure 5. A: taxol; B: docetaxel.

the establishment of the Natural Products Branch of the National Cancer Institute, with Dr. Hartwell as the first Branch Chief.

Podophyllotoxin itself proved to be too toxic for use as an anticancer agent, but an extensive program of chemical modification by workers at Sandoz, Ltd., led to the development of etoposide (Figure 4B) and teniposide (Figure 4C) as effective anticancer agents. Interestingly, unlike the parent compound, these two compounds act as inhibitors of topoisomerase II rather than of tubulin polymerization. Etoposide (Figure 4B) is used in combination with cisplatin against small-cell cancer and is also effective for the treatment of testicular cancer and non-small-cell lung cancer. Teniposide (Figure 4C) is used in combination with cisplatin against neuroblastoma, with ara-C against acute lymphoblastic leukemia, and with carboplatin against small-cell lung cancer. One of the problems with these compounds is their lack of water solubility, and the soluble compound Etopophos (Figure 4D) was developed to circumvent this problem; it was approved for clinical use in 1996. For a recent general review of the podophyllotoxins, see Lee and Xiao (2005).

Taxol

Taxol (paclitaxel, Figure 5A) and its semisynthetic analog docetaxel (Figure 5B) are two of the most important anticancer agents of the last 25 years. Taxol was originally isolated by Wall and Wani from *Taxus brevifolia* Nutt. (Wani et al., 1971), and docetaxel was prepared as a semisynthetic analog by Potier and his collaborators (Guèritte-Voegelein et al., 1986). Paclitaxel is an effective anticancer agent for breast and ovarian cancer and is also used in stents to prevent restenosis. It is a major drug, with sales of over $1.5 billion in 2000, and docetaxel is also a major drug. Some of the newer aspects of its mechanism of action are discussed later in this chapter, so this discussion has been abbreviated. For a recent general review of taxol and its analogs, see Kingston (2005).

The *Vinca* Alkaloids

The antitumor alkaloid vinblastine (Figure 6A) was isolated from *Catharanthus roseus* (L.) G. Don, formerly known as *Vinca rosea* L., by two inde-

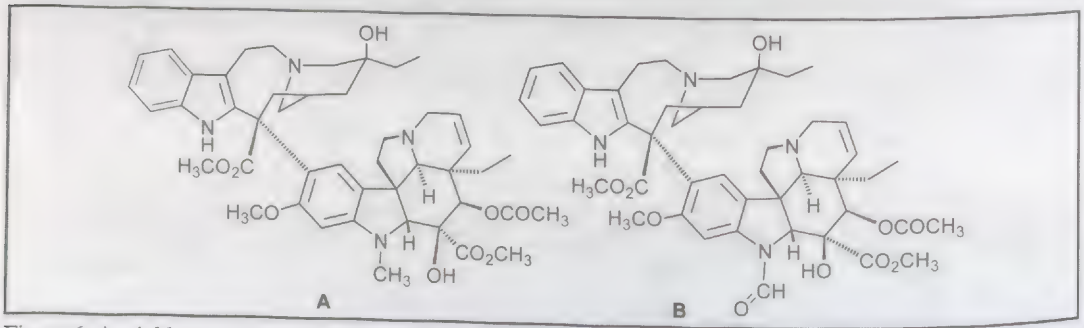

Figure 6. A: vinblastine; B: vincristine.

pendent teams during the 1950s (Noble, Beer, and Cutts, 1958; Neuss et al., 1959). The plant is known as the Madagascar periwinkle, but it is now a widespread perennial, and the actual plants collected for investigation were from Jamaica and the U.S., respectively. The closely related alkaloid vincristine (Figure 6B) and many other indole alkaloids were also isolated from the same plant (Svoboda, 1961). These alkaloids act as antimitotic agents by inhibiting the polymerization of tubulin to microtubules. Vinblastine is used in combination with other agents for treatment of Hodgkin's disease and bladder and breast cancers, while vincristine is used for treatment of acute lymphocytic leukemias and lymphomas. One of the author's Ph.D. students was cured of Hodgkin's disease by treatment with vinblastine-based chemotherapy. These compounds are also historically important as the first natural products to enter into wide clinical usage as anticancer agents. For a recent general review of the *Vinca* alkaloids, see Guèritte and Fahy (2005).

NATURAL PRODUCTS AS DRUG LEADS

As documented in the preceding section, plant secondary metabolites have provided several significant drugs for cancer chemotherapy. However, recent changes within the pharmaceutical industry have resulted in a de-emphasis of natural products research in favor of newer approaches such as combinatorial chemistry, and so it is worth commenting here on the potential of plant and other natural products for future drug discovery.

The need for new drugs is, if anything, greater now than it has ever been. The current global population is 6.1 billion, but by 2050 it will be 9.1 billion. Sadly, drug resistance is increasing at the same time as new diseases are emerging, and the cost to develop and market new drugs is increasing, with estimates ranging from $500 to $800 million for each new drug.

With this urgent scenario, it is important that we do not lose the potential drugs that can be developed from natural products. Natural products can not only provide new drug substances, they can also serve as templates for the development of new semisynthetic or totally synthetic drugs. The examples of camptothecin and the podophyllotoxins discussed earlier indicate that the best drug may not be the actual natural product, and the case of taxol discussed below will show that even a clinically useful natural product can be improved on by careful medicinal chemistry. Finally, although natural products have been explored moderately well as anticancer and anti-infective agents, their use for the treatment of many other important diseases is largely untapped.

The de-emphasis of natural products research in favor of combinatorial chemistry approaches to drug discovery has not been a roaring success, as stated recently by Ortholand and Ganesan (2004).

> The early years of combinatorial chemistry suffered from an excess of hype, and a major victim was natural-product screening. Many organizations went through an irreversible shift in policy, and prematurely discontinued their efforts in this area. We are now seeing the backlash from this knee-jerk reaction. The early combinatorial strategies were flawed and unproven, and have yet to deliver any blockbuster drugs. Meanwhile, we have lost the uniqueness of screening natural-product space as a complement to synthetic compounds. If past indicators are any guide, there are undoubtedly many more unique and potent biologically active natural products waiting to be discovered.

The importance of natural products in drug development has been emphasized in several reviews. As one example, Butler (2004), writing from the perspective of a small pharmaceutical company that is heavily invested in natural products, writes:

Another misconception has been that NP research has failed to deliver many new compounds that have undergone clinical evaluation over the last few years. However, in reality, 15 NP-derived drugs have been launched in the key markets of the United States, Europe, and Japan over the last three years, and an additional 15 NP-derived compounds were in phase III clinical trials at the end of 2003.

In summary, natural products, from whatever source, are needed to provide sources of complex and stereochemically defined "drug-like" compounds for pharmaceutical development, and although the near term may prove to be difficult for researchers in this area, the long-term prospects remain good. Several small companies have been formed to take up the challenge of drug discovery from natural products, including Galileo Pharmaceuticals and SelectX Pharmaceuticals in the U.S., Thallion Pharmaceuticals in Canada, and MerLion Pharma in Singapore. It is likely that one or more of these or other similar companies will develop an important new drug from a natural product, which will in turn increase the overall level of interest in natural products.

TAXOL: HISTORICAL INTRODUCTION

The discovery and early development of taxol (Figure 5A) have been covered in a review by Suffness and Wall (1995), and so this discussion will briefly summarize some of the more recent aspects of its development. After excellent phase II clinical results were obtained in ovarian cancer (McGuire et al., 1989) and breast cancer (Holmes et al., 1991), it became clear that taxol was destined to be a clinically important drug. At this time, in the late 1980s, essentially all the work on taxol had been carried out with National Cancer Institute (NCI) funding, but major additional investments would be needed to bring taxol successfully to market. The NCI thus invited the pharmaceutical industry to submit proposals for a Cooperative Research and Development Agreement (CRADA) that would allow the successful bidder to acquire exclusive rights to market taxol for seven years in the U.S. in exchange for investment in its development. Bristol-Myers Squibb (BMS) was selected as the CRADA partner in 1989 and immediately began a large and ultimately successful effort to develop a large-scale supply of the drug. This supply initially came from collections of *Taxus brevifolia* bark, collected primarily in the Pacific Northwest, but later a semisynthetic approach from the readily available 10-deacetylbaccatin III was developed (Kingston, 2005). Recently, BMS has moved to a plant tissue culture process for the preparation of taxol (Leistner, 2005; Wink et al., 2005).

Taxol was approved by the FDA in December 1992 for clinical use against ovarian cancer. Clinical activity against breast cancer was first reported in 1991, and it was approved for treatment of this disease in April 1994. The related compound docetaxel (Figure 5B) was discovered by Potier's group in 1986 (Guèritte-Voegelein et al., 1986), and it too has been approved by the FDA for clinical use. Taxol sales by BMS were approximately $1 billion in 2000, but these figures have declined in recent years as generic versions have become available and prices have consequently declined significantly.

TAXOL CHEMISTRY: THE HYDROXYL GROUP QUESTION

The chemistry of taxol has been studied intensively by many groups for almost 20 years, and in some cases longer, and so there is an enormous body of work in the literature on this subject. Some recent reviews are those of Kingston et al. (2002), Wang, Itokawa, and Lee (2003), Wang, Ohtsu, and Itokawa (2003), Fang and Liang (2005), Geney, Chen, and Ojima (2005), Kingston (2005), and Zhang, Zhang, and Yang (2005). This review will thus simply illustrate the work that has been done by discussing a limited number of examples from the author's own work, with a focus on the effect of removing or modifying the hydroxyl and

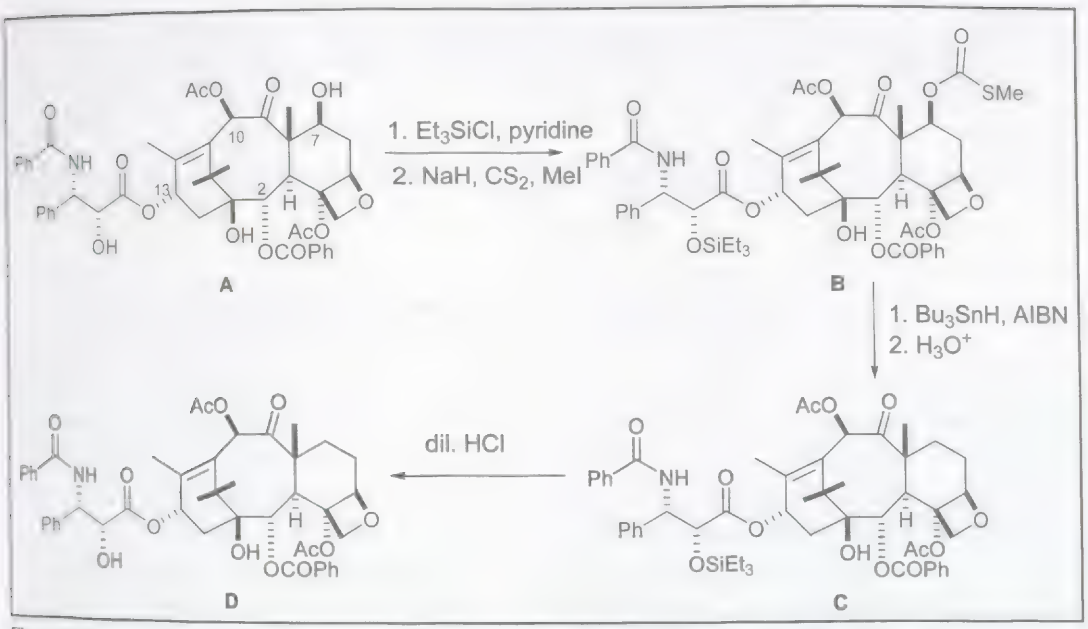

Figure 7. A: taxol; B: 7-xanthate derivative; C: 7-deoxy derivative; D: 7-deoxytaxol.

acyloxy groups that decorate taxol's baccatin III skeleton.

Taxol consists of a tetracyclic baccatin III ring system to which is appended an N-benzoylphenylisoserine side chain at C13, acyl groups at C2, C4, and C10, a hydroxyl group at C7, and a carbonyl group at C9. Early work by Wani et al. (1971) showed that the C13 side chain was necessary for activity, but the importance of the other oxygen functions was unknown. We thus launched a research program to examine the effect of each of the other oxygen functions by deleting each one in turn. The general approach we used is illustrated by the deoxygenation of the C7 hydroxyl group (Figure 7). Protection of taxol (Figure 7A) as its 2′-(triethylsilyl) ether, followed by reaction with carbon disulfide and methyl iodide in the presence of base gave the xanthate (Figure 7B). Barton deoxygenation of the xanthate gave the 7-deoxy derivative (Figure 7C), and this was deprotected to give the 7-deoxytaxol (Figure 7D) (Chaudhary, Rimoldi, and Kingston, 1993). This last compound (Figure 7D) was as cytotoxic as taxol, indicating that the C7 hydroxyl group is not necessary for activity (Chen et al., 1993).

Similar studies were carried out to prepare 10-deacetoxytaxol (Figure 8A) (Chaudhary and Kingston, 1993), 4-deacetyltaxol (Figure 8B) (Neidigh et al., 1994), and 4-deacetoxytaxol (Figure 8C) (Chordia et al., 1994). Interestingly, while 10-deacetoxytaxol (Figure 8A) had a comparable cytotoxicity to taxol, the 4-deacetyl and 4-deacetoxy compounds (Figures 8B and 8C) were both much less cytotoxic than taxol.

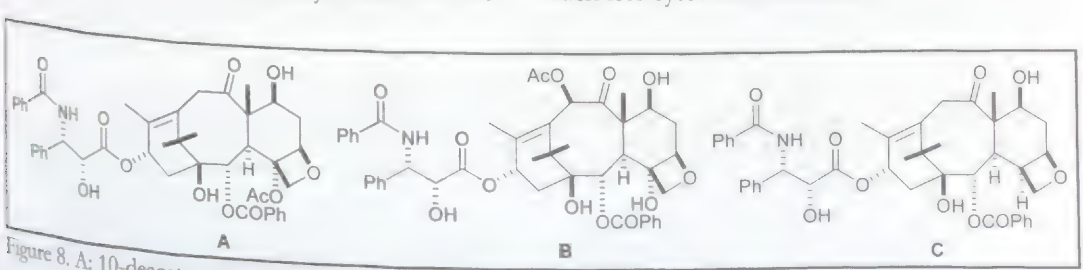

Figure 8. A: 10-deacetoxytaxol; B: 4-deacetyltaxol; C: 4-deacetoxytaxol.

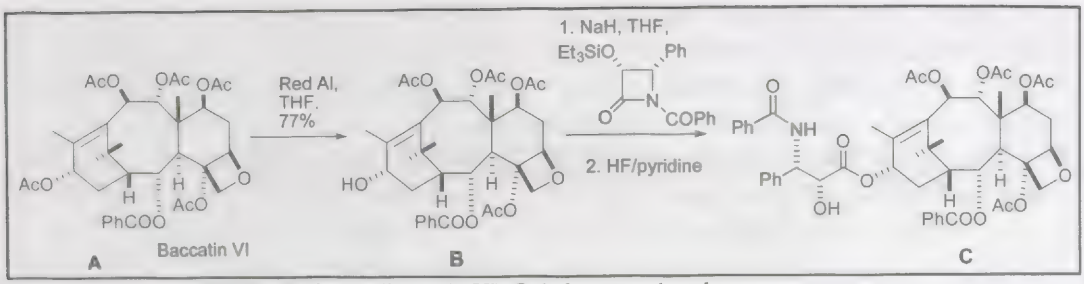

Figure 9. A: baccatin VI; B: 13-deacetylbaccatin VI; C: 1-deoxytaxol analog.

Attempted preparation of 1-deoxytaxol by the same Barton deoxygenation route led to a rearranged product (Chaudhary, Chordia, and Kingston, 1995), but the closely related compound (Figure 9C) was prepared from baccatin VI (Figure 9A) by the route shown in Figure 9 (Kingston et al., 1999). The resulting compound (Figure 9C) was approximately half as potent as taxol in a tubulin-assembly assay, and it was about one tenth as active as taxol to HCT116 colon carcinoma cells. A comparable 1-hydroxy compound had similar activity to taxol, so it appears that removal of the 1-hydroxyl group is deleterious to cytotoxicity.

Removal of the C2-benzoyl group and replacement with substituted benzoyl groups led to the exciting finding that the nature of the substituent on the benzene ring had a critically important effect on cytotoxicity and tubulin-assembly activity (Figure 10). The derivatives were prepared by careful hydrolysis of a protected taxol, to give the protected 2-debenzoyltaxol (Figure 10A). This was then reacylated with selected substituted benzoic acids to give variously substituted derivatives, and it was found that all *p*-substituted analogs (such as Figure 10B) were either inactive or much less active than taxol. Most *m*-substituted derivatives (such as Figure 10C) were, however, more active than taxol (Kingston et al., 1998).

Similar investigations by others have led to the development of several new taxol analogs that are in clinical trials, in addition to the previously described clinically used derivative docetaxel (Figure 5B). Some of these compounds are shown in Figure 11.

Although Ortataxel (Spectrum Pharmaceuticals, Irvine, CA, Figure 11A) was discontinued by its initial developer (Baumann, 2005), it is currently in phase II clinical trials by Spectrum Pharmaceuticals. RPR-109881A (Figure 11B) has been stated to have good therapeutic potential against brain tumors and drug-resistant tumors (Ojima and Geney, 2003a). The C4-carbonate BMS-188797 (Figure 11C) gave objective responses in a phase I trial in four patients, with three complete remissions in ovarian and cervical cancer patients (du Bois et al., 2006). The interesting analog DJ-

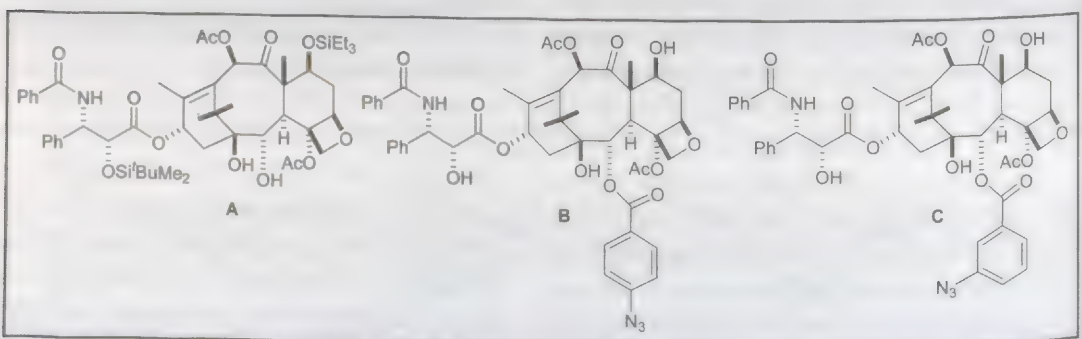

Figure 10. A: 2-debenzoyl-2'-TBDMS-taxol; B: 2-(*p*-azidobenzoyl)taxol; C: 2-(*m*-azidobenzoyl)taxol.

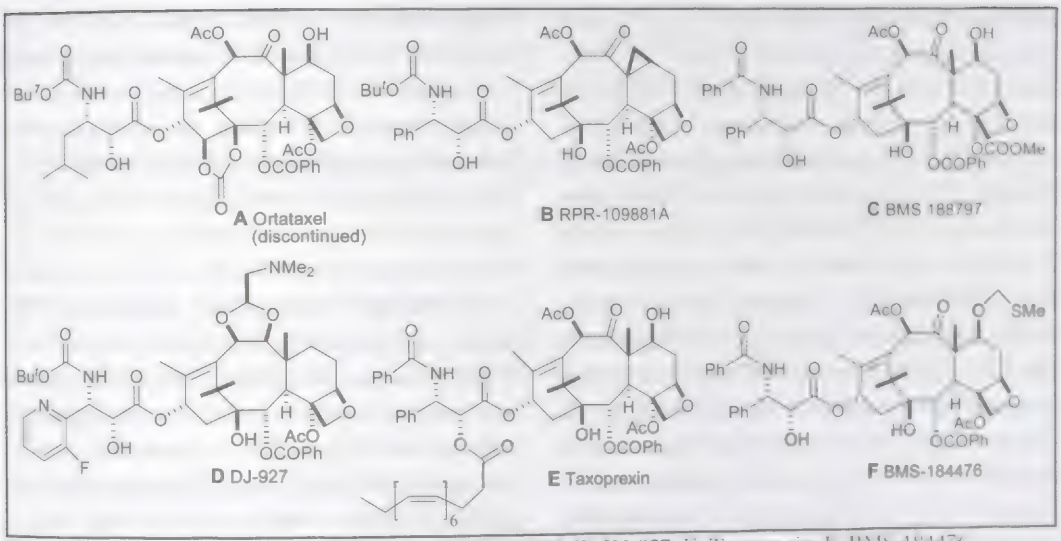

Figure 11. A: Ortataxel; B: RPR-109881A; C: BMS-188797; D: DJ-927; E: Taxoprexin; F: BMS-184476.

927 (Figure 11D), with a fluorinated pyridine ring in place of the side chain phenyl of taxol, is in phase I trials (Shionoya et al., 2003). The targeted analog Taxoprexin (Luitpold Pharmaceuticals, Inc., Shirley, NY; Figure 11E) is in phase III trials, while the thioether BMS-184476 (Figure 11F) is in phase II trials and has shown activity against previously treated non-small-cell lung cancer (Ojima and Geney, 2003b; Camps et al., 2005).

A partial summary of the structure-activity relationships of taxol is given in Figure 12. This figure summarizes most of the work described above and incorporates other results from the author's laboratory as well as from several other laboratories. Even so, it only scratches the surface of the enormous amount of work that has been done in this area, and the reader is referred to the previously cited reviews for more details.

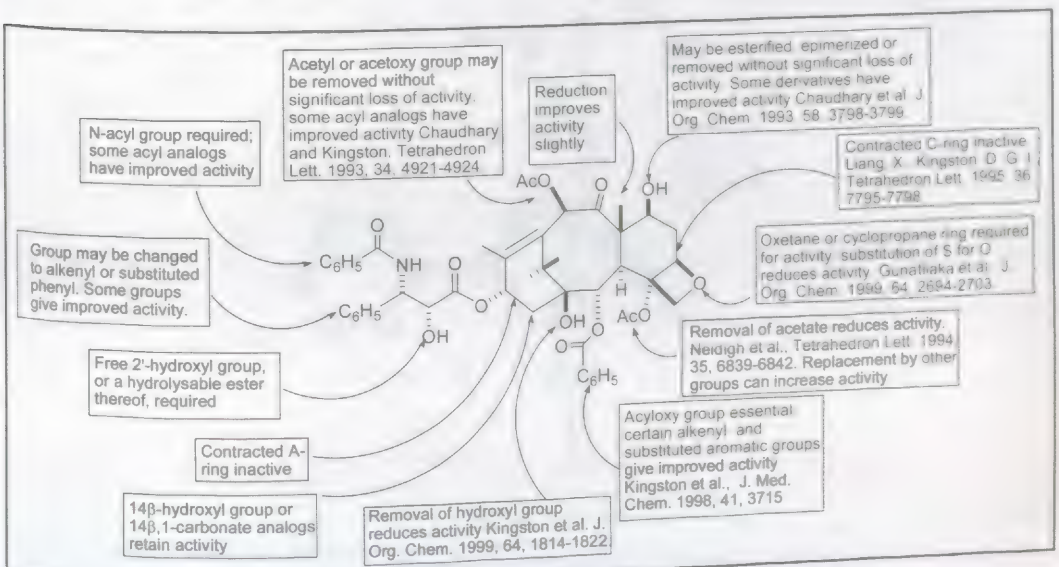
N-acyl group required; some acyl analogs have improved activity
Acetyl or acetoxy group may be removed without significant loss of activity. some acyl analogs have improved activity Chaudhary and Kingston. Tetrahedron Lett. 1993, 34, 4921-4924
Reduction improves activity slightly
May be esterified, epimerized or removed without significant loss of activity. Some derivatives have improved activity Chaudhary et al. J. Org. Chem. 1993, 58, 3798-3799
Contracted C-ring inactive Liang, X., Kingston, D. G. I. Tetrahedron Lett. 1995, 36, 7795-7798
Group may be changed to alkenyl or substituted phenyl. Some groups give improved activity.
Oxetane or cyclopropane ring required for activity; substitution of S for O reduces activity. Gunatilaka et al. J. Org. Chem. 1999, 64, 2694-2703
Removal of acetate reduces activity. Neidigh et al., Tetrahedron Lett. 1994, 35, 6839-6842. Replacement by other groups can increase activity
Free 2'-hydroxyl group, or a hydrolysable ester thereof, required
Acyloxy group essential, certain alkenyl and substituted aromatic groups give improved activity Kingston et al., J. Med. Chem. 1998, 41, 3715
Contracted A-ring inactive
14β-hydroxyl group or 14β,1-carbonate analogs retain activity
Removal of hydroxyl group reduces activity Kingston et al. J. Org. Chem. 1999, 64, 1814-1822

Figure 12. Partial summary of the structure-activity relationships of taxol.

THE TAXOL-TUBULIN INTERACTION

One of the most important discoveries in the development of taxol was Susan Horwitz's finding that taxol acts as an anticancer agent primarily through its interaction with tubulin (Schiff, Fant, and Horwitz, 1979). Tubulin, which occurs in α and β forms, is a key protein for the mitotic process. It can assemble into hollow rod-like microtubules, and these polymeric proteins provide the structure necessary for the movement of the chromosomes and ultimately the establishment of a daughter cell. The equilibrium between tubulin and microtubules is thus crucial for proper cellular function, and it is this equilibrium that is disrupted by taxol. Unlike most other antimitotic drugs such as the *Vinca* alkaloids, taxol promotes the assembly of tubulin into microtubules and stabilizes these polymers, leading ultimately to apoptosis. It turns out that fairly high concentrations of taxol are required to do this, and it exerts its therapeutic effect at significantly lower concentrations, so it has been suggested that its major effect is in disrupting the tubulin-microtubule equilibrium (Jordan and Wilson, 2004).

Since the interaction of taxol with tubulin is so important to its anticancer activity, an understanding of this interaction would provide an insight into its pharmacophore. In addition, knowledge of the binding conformation(s) of taxol could lead to the design of improved analogs. Finally, if the key reason for taxol's activity is that it fits a binding site on tubulin, a knowledge of the conformation of tubulin-bound taxol might lead to the design of simpler analogs that retain taxol's tubulin-binding activity. Unfortunately, the polymeric and non-crystalline nature of the tubulin-taxol complex makes a direct approach by X-ray crystallography unfeasible, and so various indirect methods must be used. It can

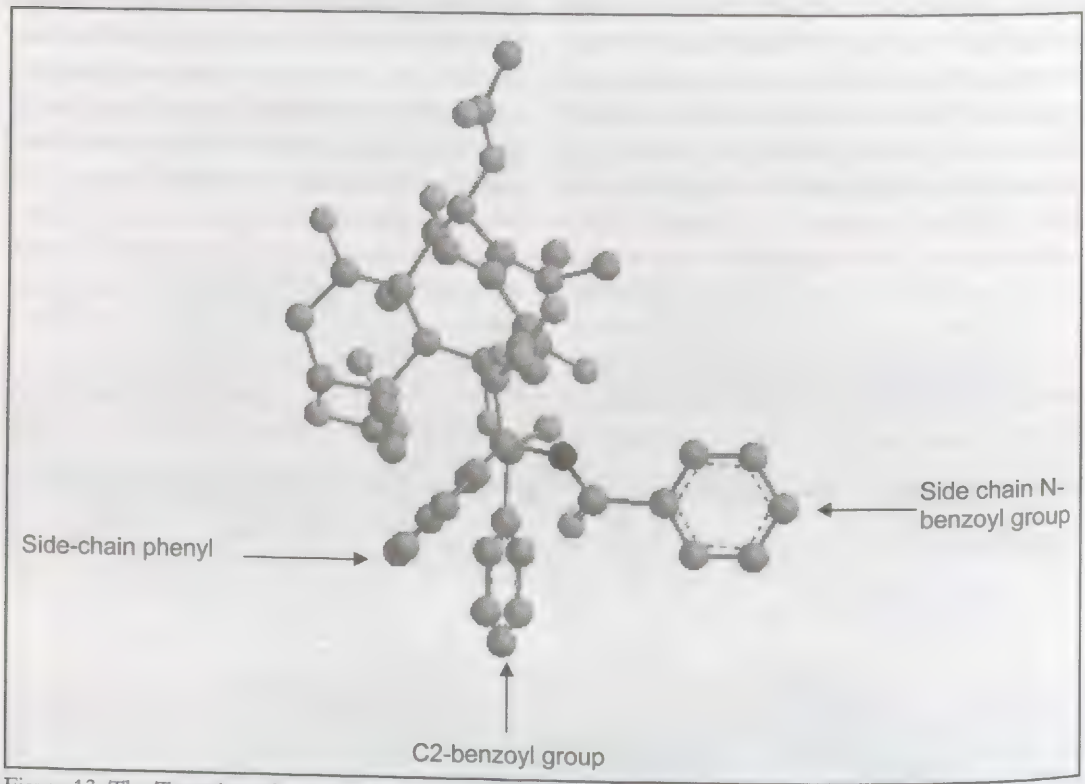

Figure 13. The T-taxol conformation, showing the C2-benzoyl group positioned between the two side chain phenyl groups.

be noted that Lowe et al. (2001) have succeeded in determining the structure of tubulin at 3.5 Å resolution, and this work forms the basis of all subsequent studies of the taxol-tubulin interaction.

One starting point for studies of the tubulin-binding conformation of taxol has been the study of its solution conformation by nuclear magnetic resonance (NMR). In non-polar solvents, taxol exists primarily in a "non-polar" conformation in which the N-benzoyl and 2-benzoyl groups are associated (Dubois et al., 1993; Williams et al., 1994). In polar aqueous solvents it adopts a set of "polar" or "hydrophobic collapsed" conformations in which the 3'-phenyl group is oriented toward the 2-benzoyl group (Vander Velde et al., 1993; Paloma et al., 1994; Ojima et al., 1999). More recently, the T-taxol conformation (Figure 13) was proposed by Snyder et al. (2001) based on the electron crystallographic structure of the tubulin-taxol complex combined with molecular dynamics and NAMFIS (NMR analysis of molecular flexibility in solution) analysis of taxol. In this conformation, the two side chain phenyl groups are approximately equidistant from the C2-benzoyl group, giving the conformer a "T-shaped" appearance.

A direct approach to answering the question of which of these proposed conformations best represents the tubulin-binding conformation of taxol is available by the technique of rotational-echo, double-resonance (REDOR) NMR spectroscopy. This technique can be applied to solids spinning at the magic angle, so it can be used for ligand-bound microtubules, and it provides a direct measurement of heteronuclear dipolar coupling between pairs of labeled nuclei. It can be very accurate, with distances of up to 12 Å capable of being determined with 0.2 Å accuracy, but it requires the synthesis of labeled ligands. In conjunction with Professors Bane at SUNY Binghamton and Schaefer at Washington University, we applied this technique to the taxol-tubulin interaction. The three labeled taxols (Figures 14A, 14B, and 14C) were prepared and were used to assemble tubulin into microtubules.

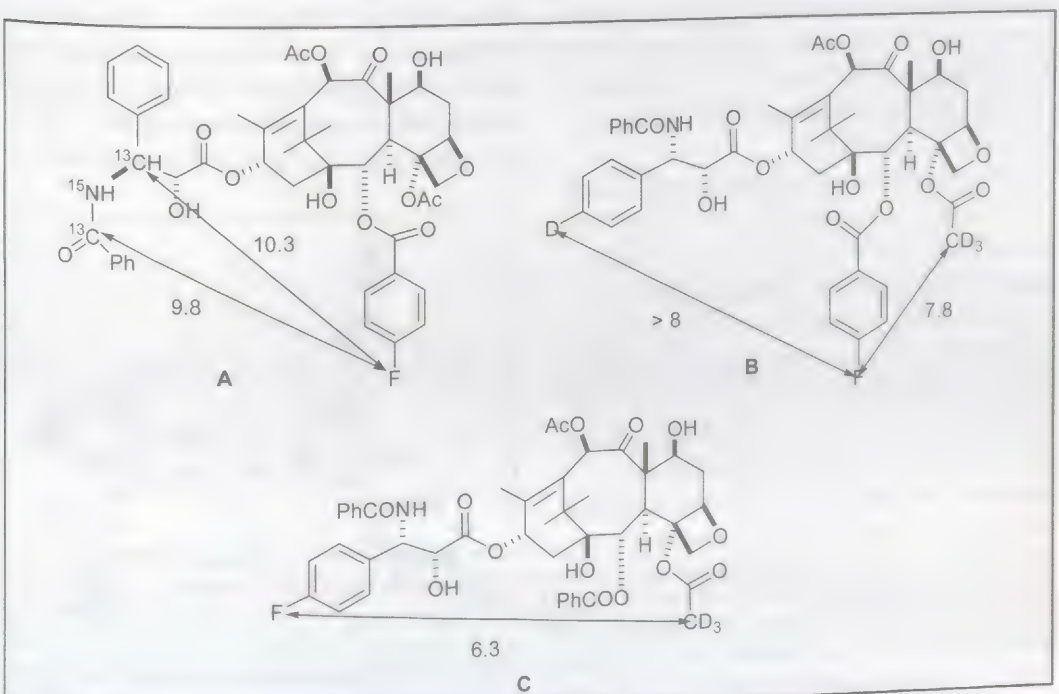

Figure 14. A–C: Key internuclear distances for three labeled taxols.

These taxol-tubulin complexes were then subjected to REDOR NMR. In the case of compound 14A,[1] 8 million scans were required to determine the distances between the fluorine on the C2-benzoyl group and the labeled side chain carbons as 9.8 and 10.3 Å (Li et al., 2000). In later work the labeled analogs (Figures 14B and 14C) were studied in the same way. Compound 14B gave a distance of 7.8 Å for the distance between the methyl group of the C4-acetate and a *p*-fluorine on the C2-benzoyl group, while compound 14C revealed a distance of 6.3 Å between the *p*-fluorine on the C3'-phenyl and the methyl protons of the C4-acetate (Paik et al., 2007). These data are fully consistent with the T-taxol hypothesis, which has a particularly short C3'-phenyl to C4-acetate distance (Figure 14).

The REDOR NMR studies described above gave strong support for the T-taxol conformation as being the best descriptor of the tubulin-binding form of taxol. Ojima and his colleagues have proposed a similar structure based on our original REDOR NMR data (Geney et al., 2005), but a detailed analysis indicates that the T-taxol model provides the most satisfying fit to all the REDOR data (Paik et al., 2007) and also to the observed electron crystallographic structure (Johnson, Alcaraz, and Snyder, 2005).

Since taxol has several rotatable substituents on its tetracyclic core structure, it is likely that its relatively weak binding to tubulin is caused in part by the existence of many conformers in solution, leading to the entropic cost of freezing the molecule out into one specific set of binding conformations. If this is the case, then it should be possible to pay this entropic cost in advance by the synthesis of a bridged taxol in the right conformation, thus improving the compound's binding to tubulin and hence its bioactivity. Several workers have prepared bridged taxol analogs by a variety of elegant approaches with the intention of preparing a more active analog, but most of these approaches were based on the polar or nonpolar conformations and yielded compounds that were much less active than the parent compound (Kingston, Bane, and Snyder, 2005). Two recent studies report the synthesis of two bridged taxanes with microtubule stabilizing capacity equivalent to taxol, but with diminished cytotoxicity (Querolle et al., 2004; Geney et al., 2005).

If the T-taxol conformation is the correct one, then it should be possible to test it experimentally by the synthesis of taxol analogs with short bridges between the C4-acetate and the C3'-phenyl positions, since these are in close juxtaposition in T-taxol (Figure 15).

The first synthetic approach was made to the *meta*-phenyl bridged compounds (Figures 16A and 16B), which were prepared by making use of the well-established Grubbs' olefin metathesis reaction on an open-chain divinyl precursor. Compound 16A can be given the trivial name *m*-britaxel-8, where the suffix 8 designates the number of atoms in the chain between the *meta* position on the C3'-phenyl and the C4 position; compound 16B can be named *m*-britaxel-6. Disappointingly, both compounds 16A and 16B were at least tenfold less active than taxol in both cytotoxicity and tubulin as-

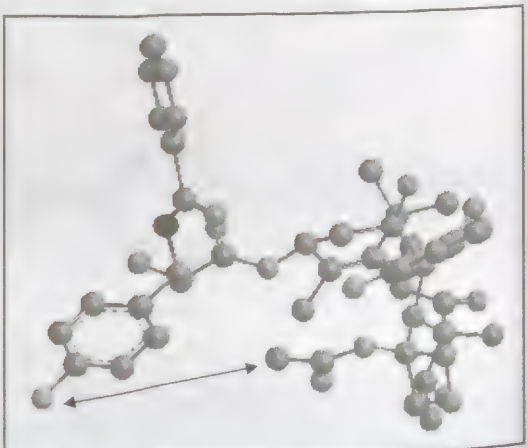

Figure 15. The T taxol conformation, showing the close approach of the C4-acetyl methyl group to a substituent in the *p*-position of the C3'-phenyl ring (arrow).

[1]In this paper, compounds shown in the figures will be referred to by the figure number and part, e.g., the compound shown in Figure 14A will be referred to as "compound 14A."

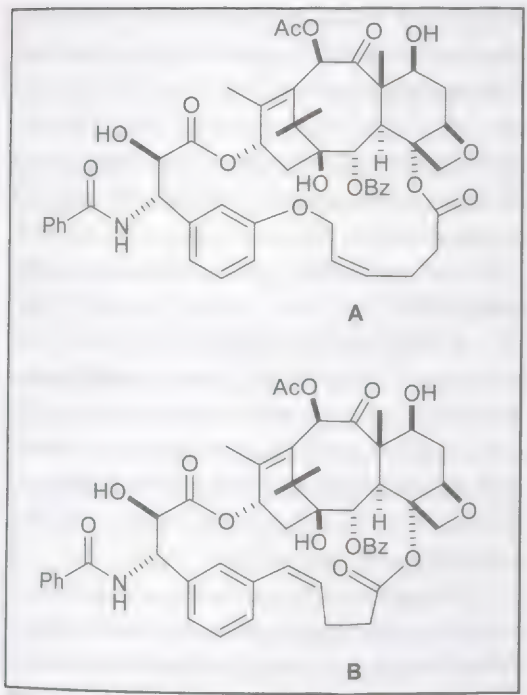

Figure 16. A and B: *Meta*-phenyl bridged compounds.

sembly assays (Metaferia et al., 2001). However, this work also showed that the reason for the lack of activity was that the best T-form of the compound 16A is seated higher than taxol in the pocket as a result of close contact between the propene moiety of the tether and Phe272 of the protein (Metaferia et al., 2001). This analysis also suggested that an *ortho*-bridged analog would not encounter this contact and should thus fit snugly into the pocket.

This prediction was confirmed by the synthesis of *o*-britaxel-5 (Figure 17H) (Ganesh et al., 2004). The β-lactam (Figure 17D) was prepared from aldehyde (Figure 17A) by established procedures, and this was coupled with the modified baccatin (Figure 17F) to yield the divinyl derivative (Figure 17G). Grubbs' olefin metathesis of 17G, followed by deprotection, gave *o*-britaxel-5 (Figure 17H).

Compound 17H was up to 20 times more active than taxol, depending on the assay used (Ganesh et al., 2004), and it was also much more ac-

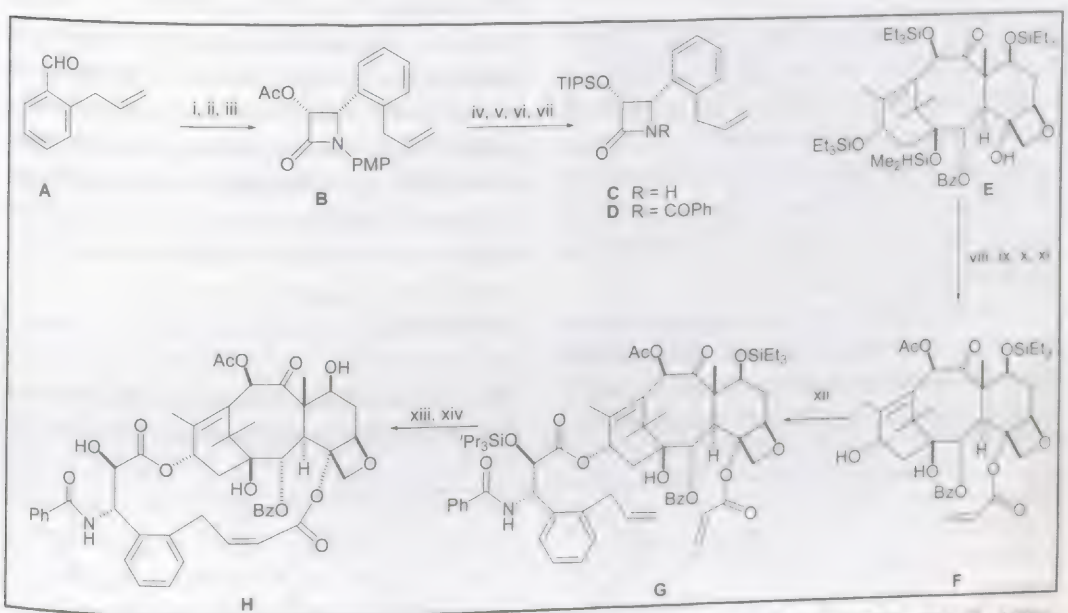

Figure 17. A–H: Synthetic scheme for the bridged paclitaxel H. i: 5b, *p*-MeOC$_6$H$_4$NH$_2$, MgSO$_4$, CH$_2$Cl$_2$, 100%; ii: CH$_3$COOCH$_2$COCl, Et$_3$N, −78°C to rt, 12 h, 85%; iii: lipase (Amano PS), phosphate buffer, pH 7.2, CH$_3$CN, 24 h, 98%; iv: 1M KOH, THF, 0°C, 100%; v: TIPSCl, imidazole, DMF, 94%; vi: CAN, CH$_3$CN, −5°C, 62%; vii: PhCOCl, Et$_3$N, DMAP, CH$_2$Cl$_2$, 95%; viii: LiHMDS, THF, 0°C, CH$_2$ = CHCOCl, 52%; ix: HF-pyridine, THF, 70%; x: CeCl$_3$, Ac$_2$O, THF, 96%; xi: Et$_3$SiCl, imidazole, DCM, 72%; xii: D, NaH, THF, 0°C to rt, 24 h; xiii: ((Cy$_3$P)(H$_2$IMes)Cl$_2$Ru = CHPh, CH$_2$Cl$_2$, 3 h; xiv: HF-pyridine, 12 h.

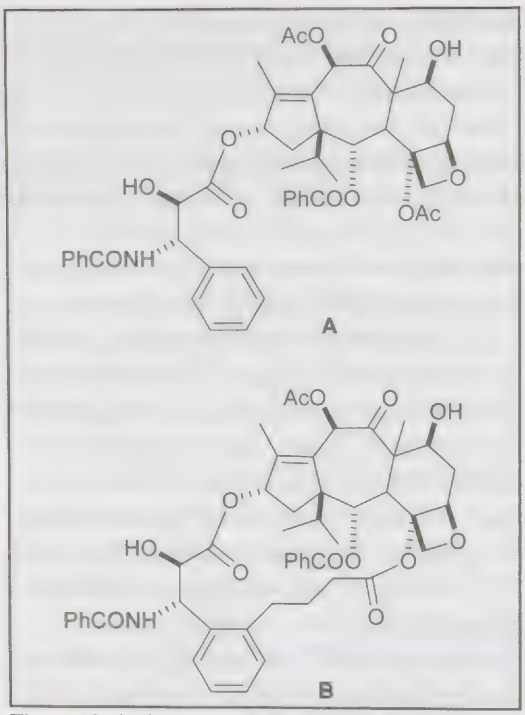

Figure 18. A: A-*nor*-taxol; B: bridged A-*nor*-taxol.

tive than taxol against two taxol-resistant cell lines and an epothilone-resistant cell line (Ganesh et al., 2007). Molecular modeling of the complex of Figure 17H with β-tubulin showed that the unfavorable interaction with Phe272 of β-tubulin had indeed been removed. These results thus served to confirm the T-taxol conformation as the correct tubulin-binding conformation of taxol.

An interesting extension of these results came from studies of A-*nor*-taxols, which showed that constraining an inactive taxol analog to the T-taxol conformation significantly increased its activity (Figure 18). We showed several years ago that the A-*nor* derivative (Figure 18A) was essentially noncytotoxic (Samaranayake et al., 1991). Synthesis of the bridged analog (Figure 18B), which was constrained to a conformation very similar to that of T-taxol, gave a product which was orders of magnitude more cytotoxic than compound 18A, although it was still less cytotoxic than taxol (Tang et al., 2006).

A final test of the ability of the T-taxol model to direct the design of bioactive taxol analogs was made by the synthesis of the greatly simplified compound 19C by coupling the β-lactam 19A and the bicyclic compound 19B, followed by Grubbs' olefin metathesis and deprotection (Figure 19) (Ganesh et al., 2006).

Compound 19C turned out to be very insoluble in mixed aqueous solvents, and it was thus difficult to obtain reliable bioassay data, but it was at least two orders of magnitude less cytotoxic than taxol to the A2780 ovarian cancer cell line. Even though this activity is modest, the overall result is an encouraging one, since this activity is significantly greater than that of compounds such as Figure 18A, which resemble taxol much more closely, and suggests that refinement of the structure of the target compound may well direct the synthesis of simplified analogs with activity comparable to or even surpassing that of taxol.

Conclusion

The preceding review has summarized one small part of the enormous amount of work that has

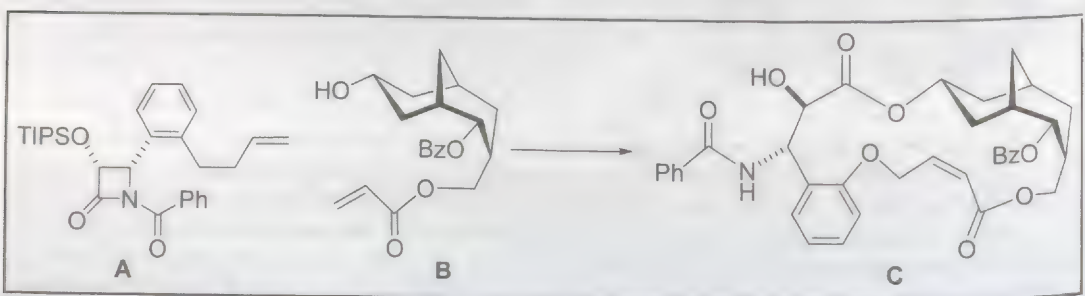

Figure 19. A: butenyl β-lactam; B: bicyclic baccatin model; C: bridged simplified taxol model.

been done on taxol. In addition to the chemical work summarized above and in the reviews previously cited, extensive studies on the clinical applications of taxol and its analog docetaxel, both alone and in combination with other drugs (Ozols, 2006; Piccart and Cardoso, 2003), have indicated that the clinical effectiveness of taxol and its sister drugs can still be improved. Taxol and its analogs have been called "the most powerful compounds" among the chemotherapeutic drugs introduced in the last decade (Nabholtz et al., 2000), but the examples of new anticancer agents of the taxane class given earlier show that improvements in this area can still be made. Improved formulations and improved drug delivery methods will also play an important role (Jaracz et al., 2005); the first new formulation has recently entered clinical use as Abraxane (Abraxis Biosciences, Los Angeles, CA), an albumin-bound form of taxol that has some advantages over the traditional Cremophor (BASF Corp., Ludwigshafen, Germany) formulation (Green et al., 2006).

Taxol analogs may also have applications in the treatment of other diseases. Thus, noncytotoxic taxanes have been suggested for treatment of tuberculosis (Huang et al., 2006). Taxol has also been shown to protect against β-amyloid toxicity in primary neurons (Michaelis et al., 1998) and to stabilize the cyclin-dependent kinase 5 activator, p35, thus decreasing β-amyloid toxicity in cortical neurons (Li et al., 2003); it is one of several newer approaches to the treatment of Alzheimer's disease (Doraiswamy and Xiong, 2006). Taxol-coated stents have also found wide application in preventing restenosis (Kamath, Barry, and Miller, 2006). The combination of new formulations with new drug targets and new chemical entities thus promises to keep taxol and its analogs at the forefront of cancer chemotherapy and probably also therapy for other diseases for many years to come.

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Bioprospecting Endophytic Microbes

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Endophytic microorganisms are to be found in virtually every higher plant on earth. These organisms reside in the living tissues of the host plant and do so in a variety of relationships ranging from symbiotic to pathogenic. Endophytes may contribute to their host plant by producing a plethora of substances that provide protection and survival value to the plant. Ultimately, these compounds, once isolated and characterized, may also have potential for use in modern medicine, agriculture, and industry. Novel antibiotics, antimycotics, immunosuppressants, and anticancer compounds are only a few examples of what has been found after the isolation and culturing of individual endophytes followed by purification and characterization of some of their natural products. Endophytic microbes represent a potential source for the discovery of new and useful compounds, or new platforms for the organic synthesis of such compounds for the benefit of mankind.

Introduction

The need for new and useful compounds to provide assistance and relief in all aspects of the human condition is ever growing. Drug resistance in disease-causing bacteria, the appearance of new, life-threatening viruses, the recurrent problems of diseases in persons with organ transplants, and the tremendous increase in the incidence of fungal infections in the world's population all underscore our inadequacy to cope with these medical problems. This is not to mention the ever-present threat of diseases that have menaced mankind from even the earliest times including malaria, tuberculosis, and diarrheal diseases. Environmental degradation, loss of biodiversity, and spoilage of land and water also add to problems facing mankind and each of these in turn can have health-related consequences. Thus, in keeping with the theme of this conference, which is to honor the work and lifelong efforts of Dr. Gordon Cragg, it seems appropriate to point out some biological niches in the world that may be harboring organisms that hold secrets in coping with some of the myriad of problems facing the world. One such small niche is the world of endophytic microbes.

Endophytes, microorganisms that reside in the tissues of living plants, are relatively unstudied as potential sources of novel natural products for use in many aspects of human activity. However, some of the most extensive and comprehensive work on natural products produced by endophytes has been done on the *Neotyphodium* sp. found on grasses (Lane, Christensen, and Miles, 2000). Alkaloids synthesized by this fungus in its grass hosts have been implicated in fescue toxicosis in rangeland animals (Lane, Christensen, and Miles, 2000). The chemistry and biology of this and other grass endophytes are reviewed elsewhere (Bacon and White, 2000). Unfortunately, because this work is so comprehensive, one may be led to the conclusion that endophytes only produce toxic compounds in their respective hosts and that they hold no promise for any medicinal, agricultural, or industrial applications. It turns out that this is simply not the case. Endophytes examined from a plethora of sources show that an overwhelming number of them produce natural products

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[2]The author expresses appreciation to the National Science Foundation, the U.S. Department of Agriculture, the National Institutes of Health, the Binational Agricultural Research and Development Foundation of Israel, the R&C Board of the State of Montana, and the Montana Agricultural Experiment Station for providing financial support for some of the work reviewed in this report. Aspects of this report have appeared in reviews, scientific reports, and book chapters in the recent past.

with promising potential for many applications. This is especially true when sourcing endophytes from higher plants existing in the world's rainforests since so much microbial diversity exists in these regions, in contrast to the limited number of plants growing in the polar regions that bear few culturable endophytes.

Within the approximately 300,000 higher plant species that exist on the earth, each individual plant, of the billions of individuals that exist, is most likely a host to one or more endophytes. Only a handful of these plants, primarily grass species, have ever been completely studied relative to their endophytic biology (Bacon and White, 2000). Consequently, the opportunity to find new and interesting endophytic microorganisms amongst the myriad of plants in different settings and ecosystems is very great. The intent of this contribution is to provide insights into the occurrence of endophytes in nature, the products that they make, and how some of these organisms are beginning to show some potential for human use. The majority of this chapter discusses rationale for study, methods used, and examples of a number of endophytes isolated and studied in the author's laboratory over the course of many years. This review, however, also includes some specific examples that illustrate the work of others in this emerging field of bioprospecting and sourcing the microbes of the world's rainforests.

The Need for and Pursuit of Microbial Products

New Medicines

There is a general call for new antibiotics, and chemotherapeutic agents that are highly effective and possess low toxicity. This search is driven by the development of resistance in infectious microorganisms (e.g., *Staphylococcus*, *Mycobacterium*, *Streptococcus*) to existing drugs and by the menacing presence of naturally resistant organisms. The ingress to the human population of disease-causing agents, such as the AIDS and Ebola viruses and the SARS coronavirus, requires the discovery and development of new drugs to combat them. Not only do diseases such as AIDS require drugs that target them specifically, but new therapies are needed for treating ancillary infections that are a consequence of a weakened immune system. Furthermore, others who are immunocompromised (e.g., cancer and organ transplant patients) are at risk of infection by opportunistic pathogens, such as *Aspergillus*, *Cryptococcus*, and *Candida*, which normally are not major problems in the human population. In addition, more drugs are needed to efficiently treat parasitic protozoan and nematodal infections such as malaria, leishmaniasis, trypanomiasis, and filariasis. Malaria, by itself, is more effective in claiming lives each year than any other single infectious agent with the exception of AIDS and tuberculosis (NIAID, 2001). However, the enteric diseases, as a group, claim the most lives each year of any other disease complex and, unfortunately, the victims are mostly children (NIAID, 2001).

Novel natural products, and the organisms that make them, offer opportunities for innovation in drug discovery. Exciting possibilities exist for those who are willing to venture into the wild and unexplored territories of the world to experience the excitement and thrill of engaging in the discovery of endophytes, their biology, and their potential usefulness.

It is evident that natural product–based compounds have an immense impact on modern medicine. For instance, about 40% of prescription drugs are based on them, and well over 50% of the new chemical products registered by the U.S. Food and Drug Administration (FDA) as anticancer agents, antimigraine agents, and antihypertensive agents are natural products or derivatives thereof in the time frame of 1981 to 2002 (Newman, Cragg, and Snader, 2003). Excluding biologics, between 1989 and 1995, 60%

of approved drugs and pre-new drug application (NDA) candidates were of natural origin (Grabley and Thiericke, 1999). From 1983–1994, over 60% of all approved and pre-NDA stage cancer drugs were of natural origin as were 78% of all newly approved antibacterial agents (Concepcion, Lazaro, and Hyde, 2001). Many examples abound that illustrate the value and importance of natural products from plants and microorganisms to modern civilizations. The discovery and development of taxol is a modern example of a natural product that has made an enormous impact on medicine (Wani et al., 1971; Rowinsky, Cazenave, and Donehower, 1990; Suffness, 1995).

Recently, however, natural product research efforts have lost popularity in many major drug companies and, in some cases, have been replaced entirely by combinatorial chemistry, which is the automated synthesis of structurally related small molecules. In addition, many drug companies have developed interests in making products that have a larger potential profit base than anti-infectious drugs. These synthetic compounds are ones that may provide social benefits, relieve allergenic responses, reduce the pain of arthritis, or soothe the stomach. It appears that this loss of interest in natural products is not only an economically driven decision but can also be attributed to the enormous effort that is required to rationally select a biological source, isolate active natural products, decipher their structures, and begin the long road to product development. It is also apparent that combinatorial chemistry and other synthetic chemistry methodologies revolving around certain basic chemical structures is now serving as a never-ending source of products to feed the screening robots of the drug industry. Within many large pharmaceutical companies, progress of professionals is based primarily upon numbers of compounds that can be produced and sent to the screening machines. This tends to work against the numerous steps needed even to find one compound in natural product discovery. It seems important to emphasize that the primary purpose of combinatorial chemistry should be to complement and assist the efforts of natural product drug discovery and development, not to supersede it (Grabley and Thiericke, 1999). Consequently, only a few larger companies still retain an interest in natural products chemistry.

Natural products often provide lead molecules or platform structures whose activity can be enhanced by manipulation through combinatorial and synthetic chemistry. Natural products have been the traditional pathfinder compounds with an untold diversity of chemical structures unparalleled by even the largest combinatorial libraries.

Potential Agricultural and Industrial Needs

While the needs for new medicines seem apparent, it is less obvious that there are also needs for new microbial products and processes in agriculture and industry. As an example, one of the pressing needs in agriculture is for a suitable replacement for methyl bromide as a soil sterilant. It is not only harmful to the farm operators, but it has been implicated in the degradation of the ozone layer that protects the earth from harmful ultraviolet radiation. Can an endophyte, or the bioactive natural products they produce, possibly be used to replace methyl bromide? Also, can endophytes be found that produce active products that can be used to control plant and animal diseases?

A major industrial problem is the biofouling of structural surfaces once they become wetted. A number of fungal species readily grow on structural surfaces, especially gypsum-based drywall, once it becomes wet. In fact, a major problem in New Orleans, after Hurricane Katrina, was the development of moldy structures that are still sound but rendered useless due to the growth of toxic fungi that have taken over structural surfaces. Is it possible that one or more endophytes may be found to assist in the amelioration of these problems?

ENDOPHYTIC MICROBES

It may also be true that a reduction in interest in natural products for use in drug development has happened as a result of people growing weary of dealing with the traditional sources of bioactive compounds, including plants of the temperate zones and microbes from a plethora of soil samples gathered in different parts of the world by armies of collectors. In other words, why continue to do the same thing when robots, combinatorial chemistry, and molecular biology have arrived on the scene? Furthermore, the logic and rationale for time and effort spent on drug discovery using a target-site directed approach have been overwhelming.

While combinatorial synthesis produces compounds at random, secondary metabolites, defined as low-molecular-weight compounds not required for growth in pure culture, are produced as an adaptation for specific functions in nature (Demain, 1981). Schutz points out that certain microbial metabolites seem to be characteristic of certain biotopes, both at an environmental as well as organismal level (Schutz, 2001). Accordingly, it appears that the search for novel secondary metabolites should center on organisms that inhabit unique biotopes. Thus, it behooves the investigator to carefully study and select the biological source before proceeding, rather than to take a totally random approach in selecting the source material. Careful study also indicates that organisms and their biotopes that are subjected to constant metabolic and environmental interactions should produce even more secondary metabolites (Schutz, 2001). Endophytes are microbes that inhabit such biotopes, namely higher plants, which is why they are currently considered as a wellspring of novel secondary metabolites offering the potential for exploitation of their medical benefits (Tan and Zou, 2000).

The investigator interested in exploiting endophytes should have access to, or have some expertise in, microbial taxonomy, and this includes modern molecular techniques involving sequence analyses of 16S and 18S rDNA. This technology is needed to help access the novelty and nature of the endophyte being studied. Currently, endophytes are viewed as an outstanding source of bioactive natural products because there are so many of them occupying literally millions of unique biological niches (higher plants) growing in so many unusual environments. Thus, it would appear that a myriad of biotypical factors associated with plants can be important in the selection of a plant for study. It may be the case that these factors govern which microbes are present in the plant as well as the biological activity of the products associated with these organisms.

What are endophytes? Bacon and White (2000) give an inclusive and widely accepted definition of endophytes: "Microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects." While the usual lack of adverse effect nature of endophyte occupation in plant tissue has prompted focus on symbiotic or mutualistic relationships between endophytes and their hosts, the observed biodiversity of endophytes suggests they can also be aggressive saprophytes or opportunistic pathogens.

Both fungi and bacteria are the most common microbes existing as endophytes. It would seem that other microbial forms most certainly exist in plants as endophytes such as mycoplasmas, rickettsia, and archebacteria; however, no evidence for them has yet been presented. In fact, it may be the case that the majority of microbes existing in plants are not culturable with common laboratory techniques, making their presence and role in plants even more intriguing.

The most frequently isolated endophytes are the fungi (Redlin and Carris, 1996). However, at the outset, it is important to note that the vast majority of plants have not been studied for any endophytic association. Thus, enormous opportunities exist for the recovery of novel fungal forms, including genera, biotypes, as well as species in the myriad of plants yet to be studied. Hawksworth and Rossman (1987) estimated there may be as many as 1 million different fungal species, yet

only about 100,000 have been described. As more evidence accumulates, estimates keep rising as to the actual number of fungal species. For instance, Dreyfuss and Chapela (1994) estimate there may be at least 1 million species of endophytic fungi alone. It seems obvious that endophytes are a rich and reliable source of genetic diversity and may represent many previously undescribed species.

Finally, in our experience, novel microbes (as defined at the morphological and/or molecular levels) often have novel natural products associated with them. This fact alone helps eliminate the problems of dereplication in compound discovery.

RATIONALE FOR PLANT SELECTION

It is important to understand that the methods and rationale used to guide plant selection seem to provide the best opportunities to isolate novel endophytic microorganisms at the genus, species, or biotype level. Since the number of plant species in the world is so great, creative and imaginative strategies must be used to quickly narrow the search for endophytes displaying bioactivity (Mittermeier, Myers, and Mittermeier, 2000).

A specific rationale for the collection of each plant for endophyte isolation and natural product discovery is used. Several hypotheses govern this plant selection strategy:

1. Plants from unique environmental settings, especially those with an unusual biology and possessing novel strategies for survival, are seriously considered for study;
2. Plants that have an ethnobotanical history (used by indigenous peoples) related to the specific uses or applications of interest are selected for study. These plants are chosen either by direct contact with local peoples or via local literature. Ultimately, it may be learned that the healing powers of the botanical source, in fact, have nothing to do with the natural products of the plant but are caused by an endophyte inhabiting the plant;
3. Plants that are endemic, have an unusual longevity, or have occupied a certain ancient landmass, such as Gondwanaland, are also more likely to lodge endophytes with active natural products than other plants;
4. Plants growing in areas of great biodiversity, it may follow, also have the prospect of housing endophytes with great biodiversity.

Just as plants from a distinct environmental setting are considered to be a promising source of novel endophytes and their compounds, so too are plants with an unconventional biology. For example, an aquatic plant, *Rhyncholacis penicillata* Matthiesen, was collected from a river system in southwest Venezuela, where the harsh aquatic environment subjected the plant to constant beating by virtue of rushing waters, debris, and tumbling rocks and pebbles (Strobel et al., 1999a). These environmental insults created many portals through which common phytopathogenic oomycetes could enter the plant. Still, the plant population appeared to be healthy, possibly due to protection provided by an endophytic product. This was the environmental biological clue used to pick this plant for a comprehensive study of its endophytes. Eventually, an unusual and potent antifungal strain of *Serratia marcescens*, living both as an epiphyte and an endophyte, was recovered from *R. penicillata*. This bacterium produces oocydin A, a novel antioomycetous chlorinated macrocyclic lactone (Figure 1) (Strobel et al., 1999b). It is conceivable that the production

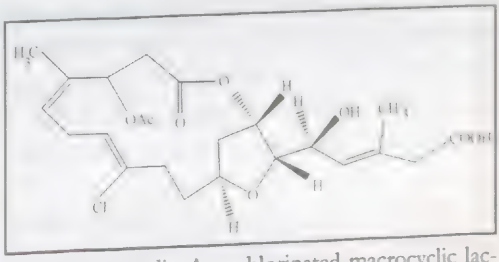

Figure 1. Oocydin A, a chlorinated macrocyclic lactone isolated and characterized from a strain of *Serratia marcescens*, obtained from *Rhyncholacis penicillata* (stereochemistry unknown).

of oocydin A by *S. marcescens* is directly related to the endophyte's relationship with its higher plant host. Currently, oocydin A is being considered for agricultural use to control the ever-threatening presence of oomycetous fungi such as *Pythium* spp. and *Phytophthora* spp. Oocydin A also has activity against a number of rapidly dividing cancer cell lines (Strobel et al., 1999b).

Plants with an ethnobotanical history are also are likely candidates for study since the medical uses for which the plant was selected may relate more to its population of endophytes than to the plant biochemistry itself. For example, a sample of the snakevine *Kennedia nigricans* Lindl., from the Northern Territory of Australia, was selected for study since its sap has traditionally been used as a bush medicine. In fact, this area was selected for plant sampling since it has been home to the world's longest-standing human civilization, the Australian Aborigines. The snakevine is harvested, crushed, and heated in an aqueous brew by local Aborigines in southwest Arnhem Land to treat cuts, wounds, and infections. As it turned out, the plant contains an entire suite of streptomycetes (Castillo et al., 2005). One in particular, designated *Streptomyces* NRRL 30562, has unique partial 16S rDNA sequences when compared to those in GenBank (Castillo et al., 2002). It produces a series of actinomycins including actinomycins D, Xo_{β}, and X_2, which will be described below. It also produces novel, broad-spectrum peptide antibiotics called munumbicins that are discussed below. A myriad of other products with antibiotic properties are also found in the culture fluids of this microbe. This appears to be an excellent example illustrating the potential that some of the healing properties in plants, as discovered by indigenous peoples, might be facilitated by compounds produced by one or more specific plant-associated endophytes as well as the plant products themselves.

Furthermore, some plants generate bioactive natural products and have associated endophytes that produce the same natural products. Such is the case with taxol, a highly functionalized diterpenoid and famed anticancer agent that is found in *Taxus brevifolia* Nutt. and other yew species (*Taxus* L. spp.) (Wani et al., 1971). In 1993, a novel taxol-producing fungus, *Taxomyces andreanae*, was isolated and characterized from *Taxus brevifolia* (Strobel et al., 1993).

Endophytes and Biodiversity

Of the myriad of ecosystems on the earth, those having the greatest general biodiversity of life seem to be the ones also having the greatest number and most diverse endophytes. Tropical rainforests and cloud forests are the most biologically diverse terrestrial ecosystems. The most threatened of these spots cover only 1.44% of the land's surface, yet they harbor over 60% of the world's terrestrial biodiversity (Mittermeier, Myers, and Mittermeier, 2000). In addition, each of the 20 to 25 areas identified as supporting the world's greatest biodiversity also support unusually high levels of plant endemism (Mittermeier, Myers, and Mittermeier, 2000). As such, one would expect, with high plant endemism, there also should exist specific endophytes that may have evolved with the endemic plant species. Biological diversity implies chemical diversity because of the constant chemical innovation that is required to survive in ecosystems where the evolutionary race to survive is most active. Tropical rainforests and tropical cloud forests are a remarkable example of this type of environment. Competition is great, resources are limited, and selection pressure is at its peak. This gives rise to a high probability that these forests are a source of novel molecular structures and biologically active compounds (Redell and Gordon, 2000).

Bills et al. (2002) describe a metabolic distinction between tropical and temperate endophytes through statistical data, which compare the number of bioactive natural products isolated from endophytes of tropical regions to

the number of those isolated from endophytes of temperate origin. Not only did they find that tropical endophytes provide more active natural products than temperate endophytes, but they also noted that a significantly higher number of tropical endophytes produced a larger number of active secondary metabolites than did fungi from other substrata. This observation suggests the importance of the host plant as well as the ecosystem in influencing the general metabolism of endophytic microbes.

Endophytes and Natural Products

It has been suggested that the reason some endophytes produce certain phytochemicals, originally characteristic of the host, might be related to a genetic combination of the endophyte with the host that occurred in evolutionary time (Tan and Zou, 2000). This is a concept that was originally proposed as a mechanism to explain why *Taxomyces andreanae* may be producing taxol (Stierle, Strobel, and Stierle, 1993). Thus, if endophytes, and especially cultured endophytes, can produce the same rare and important bioactive compounds as their host plants, this would not only reduce the need to harvest slow-growing and possibly rare plants, but also help to preserve the world's ever-diminishing biodiversity. Furthermore, it is recognized that a microbial source of a high value product may be easier and more economical to produce effectively, thereby reducing its market price.

All aspects of the biology and interrelationship of endophytes with their respective hosts is a vastly under-investigated and exciting field (Strobel, 2002a, 2002b; Strobel and Daisy, 2003; Strobel et al., 2004). Thus, more background information on a given plant species and its microorganismal biology would be exceedingly helpful in directing the search for bioactive products. Presently, no one is quite certain of the role of endophytes in nature and what appears to be their relationship to various host plant species. While some endophytic fungi appear to be ubiquitous (e.g., *Fusarium* spp., *Pestalotiopsis* spp., and *Xylaria* spp.), one cannot definitively state that endophytes are truly host-specific or even systemic within plants any more than assume that their associations are chance encounters. Frequently, many endophytes of the same species are isolated from the same plant, and only one or a few biotypes of a given fungus will produce a highly biologically active compound in culture (Li et al., 1996). A great deal of uncertainty also exists about what an endophyte produces in culture and what it may produce in nature. It does seem possible that the production of certain bioactive compounds by the endophyte in situ may facilitate the domination of its biological niche within the plant or even provide protection to the plant from harmful invading pathogens. Furthermore, little information exists relative to the biochemistry and physiology of the interactions of the endophyte with its host plant. It would seem that many factors changing in the host as related to the season, and other factors including age, environment, and location, may influence the biology of the endophyte. Indeed, further research at the molecular level must be conducted in the field to study endophyte interactions and ecology. All of these interactions are probably chemically mediated for some purpose in nature. An ecological awareness of the role these organisms play in nature will provide the best clues for targeting particular types of endophytic bioactivity with the greatest potential for bioprospecting.

Isolation, Preservation, and Storage of Endophytic Cultures for Product Isolation

Detailed techniques for the isolation of microbial endophytes are outlined in a number of reviews and technical articles (Strobel et al., 1993, 1999b, 2004; Castillo et al., 2002, 2005; Strobel, 2002a, 2002b; Strobel and Daisy, 2003).

If endophytes are being obtained from plants growing in polar regions, the dry tropics, or some temperate areas of the world, one can expect to acquire from none to only one or two endophytic cultures per plant sample (0.5–10.0 cm limb piece). However, from the wet tropics this number may rise to 20 to 30 or even more microbes per plant piece. Given limited fermentation capabilities, it is critical to label and store cultures of freshly isolated microbes for work in the future and for patent and publication purposes.

Generally, preservation in an aqueous 15% glycerol solution at −70°C is an exceedingly good procedure for saving cultures until work on them can proceed at a later date (Strobel, 2002a, 2002b; Strobel and Daisy, 2003; Strobel et al., 2004). Growing fungal cultures on sterilized barley or other grains with subsequent storage at −70°C is an effective alternative to the glycerol storage solution, and it may represent the best means to store microbes of interest for long periods of time.

Some Examples of Bioactive Natural Products from Endophytes

The following section shows some examples of natural products obtained from endophytic microbes and their potential in the pharmaceutical and agrochemical arenas. Many of the examples are taken from our own work and, thus, this review is by no means inclusive of all-natural product work in endophytes.

Endophytic Fungal Products as Antibiotics

Fungi are the most commonly isolated endophytic microbes. They usually appear as fine filaments growing from the plant material on the agar surface. Generally, the most commonly isolated fungi are in the group fungi imperfecti or Deuteromycetes. Basically, they produce asexual spores in or on various fruiting structures. Also, it is quite common to isolate endophytes that produce no fruiting structures whatsoever, such as *Mycelia sterilia.*

Quite commonly, endophytes do produce secondary metabolites when placed in culture. However, the temperature, the composition of the medium, and the degree of aeration will affect the amount and kinds of compounds that are produced by an endophytic fungus. Sometimes endophytic fungi produce antibiotics. Natural products from endophytic fungi have been observed to inhibit or kill a wide variety of harmful microorganisms including, but not limited to, phytopathogens, as well as bacteria, fungi, viruses, and protozoans that affect humans and animals.

Cryptosporiopsis cf. *quercina* is the imperfect stage of *Pezicula cinnamomea*, a fungus commonly associated with hardwood species in Europe. It was isolated as an endophyte from *Tripterygium wilfordii* Hook. f., a medicinal plant native to Eurasia (Strobel et al., 1999c). On Petri plates, *C. quercina* demonstrates excellent antifungal activity against some important human fungal pathogens including *Candida albicans* and *Trichophyton* spp. Cryptocandin, a unique peptide antimycotic related to the echinocandins and the pneumocandins (Walsh, 1992), was isolated and characterized from *C. quercina* (Strobel et al., 1999c). This compound contains a number of peculiar hydroxylated amino acids and a novel amino acid, 3-hydroxy-4-hydroxymethyl proline (Figure 2). It is generally true that not one but several bioactive and related compounds are produced by an endophytic microbe. So it is that other antifungal agents related to cryptocandin are also produced by *C. quercina.* Cryptocandin is also active against a number of plant pathogenic fungi including *Sclerotinia sclerotiorum* and *Botrytis cinerea.* Cryptocandin and its related compounds are currently being considered for use against a number of fungi-causing diseases of the skin and nails.

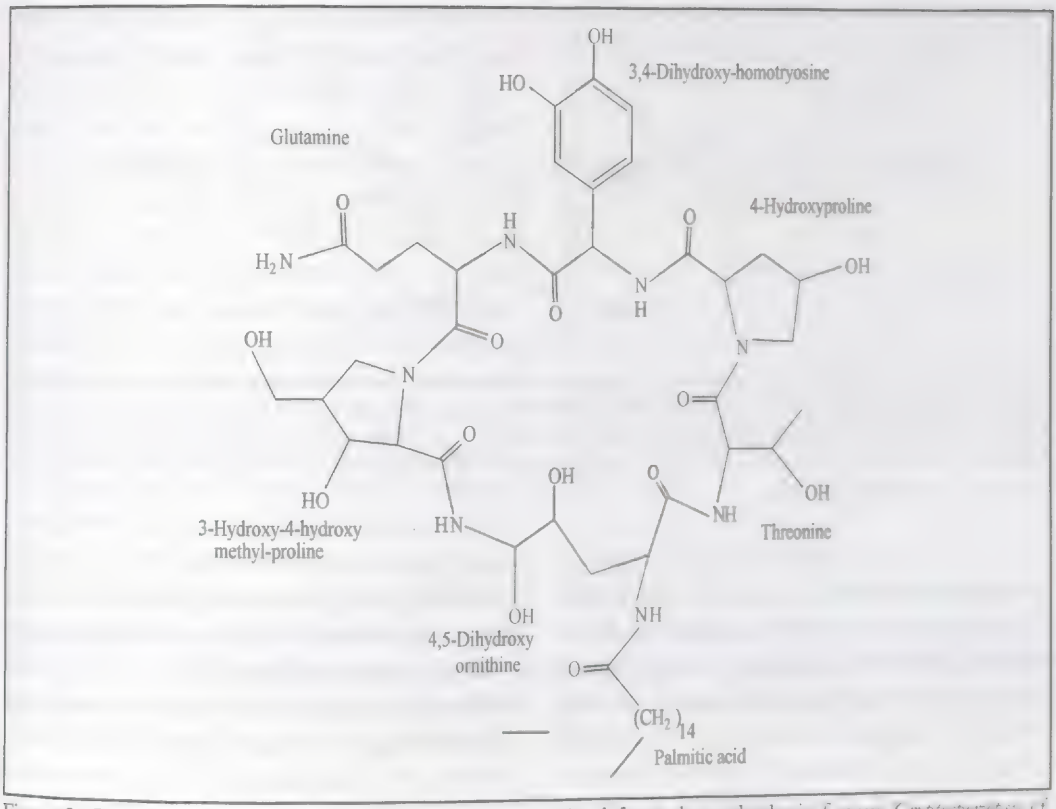

Figure 2. Cryptocandin A, an antifungal lipopeptide obtained from the endophytic fungus *Cryptosporiopsis* cf. *quercina*. (No stereochemistry is intended.)

Cryptocin, a unique tetramic acid, is also produced by *Cryptosporiopsis quercina* (Figure 3) (Li et al., 2000). This unusual compound possesses potent activity against *Pyricularia oryzae*, the causal organism of "rice blast," one of the worst plant diseases in the world, as well as a number of other plant pathogenic fungi (Li et al., 2000). The compound was generally ineffective against a general array of human pathogenic fungi. Nevertheless, with minimum inhibitory concentrations against *P. oryzae* at 0.39 μg/mL, this compound is being examined as a natural chemical control agent for rice blast and is being used as a model to synthesize other antifungal compounds.

Pestalotiopsis microspora is a common rainforest endophyte (Strobel, 2002a, 2002b; Strobel and Daisy, 2003). It turns out that enormous biochemical diversity does exist in this endophytic fungus, and many secondary metabolites are produced by various strains of this widely dispersed organism. One such secondary metabolite is ambuic acid, an antifungal agent, which has been recently described from several isolates of *P. microspora* found

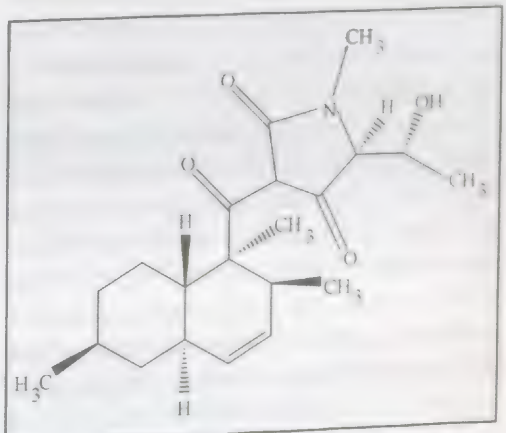

Figure 3. Cryptocin, a tetramic acid antifungal compound found in *Cryptosporiopsis* cf. *quercina*.

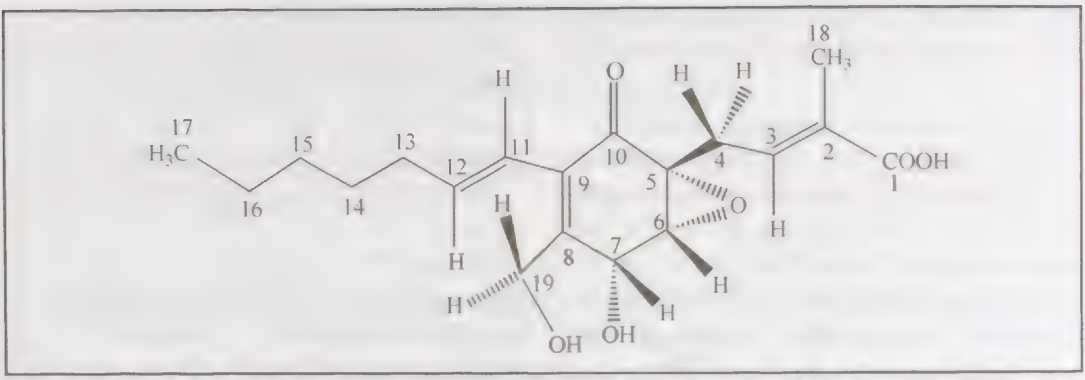

Figure 4. Ambuic acid, a highly functionalized cyclohexenone epoxide produced by a number of isolates of *Pestalotiopsis microspora* found in rainforests around the world. This compound possesses antifungal activity and has been used as a model compound for the development of solid-state NMR methods for the structural determination of natural products.

as representative isolates in many of the world's rainforests (Figure 4) (Li et al., 2001). As an interesting spin-off to endophyte studies, ambuic acid and another endophyte product, terrein, have been used as models to develop new solid-state nuclear magnetic resonance (NMR) tensor methods to assist in the characterization of molecular stereochemistry of organic molecules (Harper et al., 2000, 2001).

A strain of *Pestalotiopsis microspora* was isolated from the endangered tree *Torreya taxifolia* Arn. and produces several compounds having antifungal activity including pestaloside, an aromatic β-glucoside, and two pyrones, pestalopyrone and hydroxypestalopyrone (Lee et al., 1995b). These compounds also possess phytotoxic properties. Other newly isolated secondary products obtained from *P. microspora* (endophytic on *Taxus brevifolia*) include two new caryophyllene sesquiterpenes, pestalotiopsins A and B (Pulici et al., 1996a), 2α-hydroxydrimeninol, and a highly functionalized humulane (Pulici et al., 1996b, 1996c). Variation in the amount and kinds of products found in this fungus depends on both the culture conditions as well as the original plant source from which it was isolated.

Pestalotiopsis jesteri is a newly described endophytic fungal species from the Sepik River area of Papua New Guinea, and it produces the highly functionalized cyclohexenone epoxides, jesterone and hydroxy-jesterone, which exhibit antifungal activity against a variety of plant pathogenic fungi (Li and Strobel, 2001). Jesterone has subsequently been synthesized with complete retention of biological activity (Figure 5) (Hu et al., 2001). Jesterone is one of only a few products from endophytic microbes for which total synthesis has been successfully accomplished.

Phomopsichalasin, a metabolite from an endophytic *Phomopsis* sp., represents the first cytochalasin-type compound with a three-ring system replacing the cytochalasin macrolide ring. This metabolite exhibits antibacterial activity in

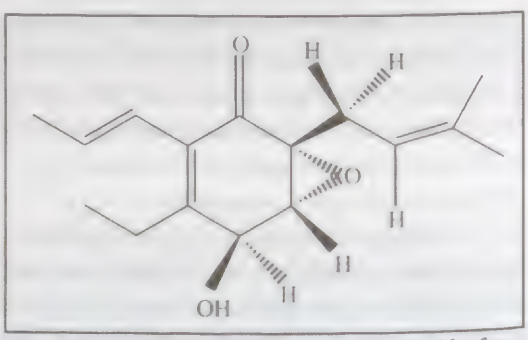

Figure 5. Jesterone, a cyclohexenone epoxide from *Pestalotiopsis jesteri,* has antioomycete activity.

disk diffusion assays (at a concentration of 4 μg/disk) against *Bacillus subtilis*, *Salmonella gallinarum*, and *Staphylococcus aureus*. It also displays a moderate activity against the yeast *Candida tropicalis* (Horn et al., 1995).

An endophytic *Fusarium* sp. isolated from *Selaginella pallescens* (C. Presl) Spring collected in the Guanacaste Conservation Area of Costa Rica was screened for antifungal activity. A new pentaketide antifungal agent, CR377, was isolated from the culture broth of the fungus and showed potent activity against *Candida albicans* in agar diffusion assays (Brady and Clardy, 2000).

Colletotric acid, a metabolite of *Colletotrichum gloeosporioides*, an endophytic fungus isolated from *Artemisia mongolica* (Fisch. ex Besser) Nakai, displays antibacterial activity against bacteria as well as against the fungus *Helminthosporium sativum* (Zou et al., 2000). Antimicrobial products have been identified from another *Colletotrichum* sp. isolated from *A. annua* L., a traditional Chinese herb that is well recognized for its synthesis of artemisinin (an antimalarial drug) and its ability to inhabit many geographically different areas. The *Colletotrichum* sp. found in *A. annua* produce not only metabolites with activity against human pathogenic fungi and bacteria, but also metabolites that caused stasis to plant pathogenic fungi (Lu et al., 2000).

A novel antibacterial agent, guignardic acid, was isolated from the endophytic fungus *Guignardia* sp. Interestingly, the organism was obtained from the medicinal plant *Spondias mombin* L. of the tropical plant family Anacardiaceae found in Brazil. The compound was isolated by UV-guided fractionation of the fermentation products of this fungus and is the first member of a novel class of natural compounds containing a dioxolanone moiety formed by the fusion of 2-oxo-3-phenylpropanoic acid and 3-methyl-2-oxobutanoic acid, which are products of the oxidative deamination of phenylalanine and valine, respectively (Figure 6) (Rodriguez-Heerklotz et al., 2001).

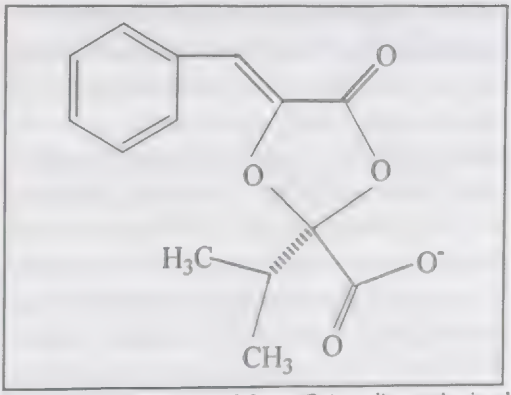

Figure 6. Guignardic acid from *Guignardia* sp. obtained from *Spondias mombin*, an anacardiaceous plant in Brazil.

Endophytic Bacterial Products as Antibiotics

There are only a limited number of bacterial genera known to be associated with plants including, among others, *Bacillus*, *Pseudomonas*, and *Streptomyces*.

Endophytic *Pseudomonas* Species

One of the most common endophytic bacterial genera encountered is *Pseudomonas*, which has representative biotypes and species that are epiphytic, endophytic, and pathogenic. They have been reported from every continent including Antarctica. Some of these species produce phytotoxic compounds as well as antibiotics.

The ecomycins, active against human pathogenic fungi such as *Cryptococcus neoformans* and *Candida albicans*, are produced by *Pseudomonas viridiflava*, which is generally associated with the leaves of many grass species and is located on and within the tissues (Miller et al., 1998). These novel lipopeptides have masses of 1,153 and 1,181 Da and contain, in addition to the common amino acids such as alanine, serine, threonine, and glycine, some unusual amino acids including homoserine and β-hydroxyaspartic acid (Miller et al., 1998).

The pseudomycins, produced by a plant-associated pseudomonad, are another group of

antifungal peptides (Harrison et al., 1991; Ballio et al., 1994). They are active against a variety of plant and human pathogenic fungi including *Candida albicans* and *Cryptococcus neoformans*, and a variety of plant pathogenic fungi including *Ceratocystis ulmi* (the Dutch elm disease pathogen) and *Mycosphaerella fijiensis* (causal agent of Black Sigatoka disease in bananas). The pseudomycins are cyclic depsipeptides that include several nontraditional amino acids including L-chlorothreonine, L-hydroxyaspartic acid, and both D and L-diaminobutryic acid. Cyclization is formed by acylation of the OH group of the N-terminal serine with the terminal carboxyl group of L-chlorothreonine. Variety in this family of compounds is imparted via N-acetylation by one of a series of fatty acids including 3,4-dihydroxydecanoic and 3-hydroxytetradecanoic acids (Ballio et al., 1994). The molecules are candidates for use in human medicine especially, since structural modification by chemical synthesis has successfully removed mammalian toxicity (Zhang et al., 2001). The pseudomycins are also effective against a number of ascomycetous fungi and are being considered for the control of the Black Sigatoka disease in bananas (Strobel, unpublished results).

Endophytic Streptomycetes Species

Streptomyces spp. are filamentous bacteria, belonging to the group Actinomycetales, that live in widely diverse ecological settings. Generally, the actinomycetes are Gram-positive organisms with a high G+C content, which do not have an organized nucleus. To date, actinomycetes have been the world's greatest source of natural antibiotics with just one genus, *Streptomyces*, the source of 80% of these compounds (Kieser et al., 2000). The majority of the antibiotic producers are known from soil sources, and, until recently, it was not realized that these organisms can exist as endophytes. One of the first endophytic *Streptomyces* spp. isolated was that from perennial ryegrass (*Lolium perenne* L.) (Guerny and Mantle, 1993). This isolate produces methylalbonoursin, a weakly antibiotic diketopiperazine (Guerny and Mantle, 1993). Other streptomycetes with activities against plant pathogens have been isolated from a number of plant species in Japan and are being considered as biocontrol agents (Kunoh, 2002).

Using the ethnobotanical approach to plant selection, the snakevine plant *Kennedia nigricans* was chosen as a possible source of endophytic microbes because of its long traditional use by Australian Aborigines to treat cuts and open wounds, resulting in reduced infection and rapid healing. This plant, collected near the Aboriginal community of Manyallaluk in the Northern Territory of Australia, consistently produced an endophytic actinomycete designated *Streptomyces* NRRL 30562 (Castillo et al., 2002). The organism was not found in several tree species supporting the vine, suggesting a host-selective or specific association of the endophyte with a specific plant genus. This streptomycete produces a family of actinomycins including D, Xo_{β}, and X_2. These compounds may not only protect the plant from fungal and bacterial infections, but also have unknowingly served the Aborigines as a source of bush medicine.

The snakevine-derived *Streptomyces* NRRL 30562 also produces the novel antibiotics munumbicins E-4 and E-5, which possess widely differing biological activities, depending on the target organism (Castillo et al., 2006). These compounds appear to be related to the actinomycins, but have a mild yellowish coloration and unique NMR spectra and possess masses of 1445. The munumbicins are effective against a wide range of fungal pathogens of plants and certain Gram-negative and Gram-positive bacteria. The compounds are currently being more completely chemically characterized.

More recently, endophytic streptomycetes have been discovered in the upper Amazon of Peru, an area of the world claimed to be one of

the most biologically diverse. The inner tissues of the "follow-me vine," *Monstera* Adans. sp., commonly yields a verticillated streptomycete with outstanding inhibitory activities against pythiaceous fungi as well as the malarial parasite *Plasmodium falciparum*. The bioactive component is a mixture of lipopeptides and named coronamycin (Ezra et al., 2004).

Volatile Antibiotics from Endophytes

Muscodor albus is a newly described endophytic fungus obtained from small limbs of *Cinnamomum zeylanicum* Blume (cinnamon tree) (Worapong et al., 2001). This xylariaceous (non-spore-producing) fungus effectively inhibits and kills certain other fungi and bacteria by producing a mixture of volatile compounds (Strobel et al., 2001). The majority of these compounds have been identified by gas chromatography (GC)/mass spectrometry (MS), synthesized or acquired, and then ultimately formulated into an artificial mixture that mimicked the antibiotic effects of the volatile compounds produced by the fungus (Strobel et al., 2001). Individually, each of the five classes of volatile compounds produced by the fungus had some antimicrobial effects against the test fungi and bacteria, but none was lethal. However, collectively they acted synergistically to cause death in a broad range of plant and human pathogenic fungi and bacteria. The most effective class of inhibitory compounds was the esters, of which isoamyl acetate was the most biologically active; however, in order to be lethal it needs to be combined with other volatiles (Strobel et al., 2001). The composition of the medium on which *M. albus* is grown dramatically influences the kind of volatile compounds that are produced (Ezra and Strobel, 2003).

The ecological implications and potential practical benefits of the "mycofumigation" effects of *Muscodor albus* are very promising given the fact that soil fumigation utilizing methyl bromide will soon be illegal in the U.S. Methyl bromide is not only a hazard to human health, but it has been implicated in causing destruction of the ozone layer. The potential use of mycofumigation to treat soil, seeds, and plants will soon be a reality as AgraQuest of Davis, California, has EPA approval to release this organism for agriculture uses. The artificial mixture of volatile compounds may also have usefulness in treating seeds, fruits, and plant parts in storage and while being transported. In addition, *M. albus* is already in a limited market for the treatment of human wastes. Its gases have both inhibitory and lethal effects on such feces-inhabiting organisms as *Escherichia coli* and *Vibrio cholera*. It has been used for this purpose starting in 2006. Studies are underway that show its promise to fumigate buildings, thus removing the potential for fungi to contaminate building surfaces and cause health risks. It is also being examined for its direct medicinal potential.

Using *Muscodor albus* as a screening tool, it has now been possible to isolate other endophytic fungi, producing volatile antibiotics. The newly described *M. roseus* was twice obtained from tree species found in the Northern Territory of Australia. This fungus is just as effective in causing inhibition and death of test microbes in the laboratory as *M. albus* (Worapong et al., 2002). Other interesting *M. albus* isolates have been obtained from several plant species growing in the Northern Territory of Australia and the jungles of the Tesso Nilo area of Sumatra, Indonesia (Ezra, Hess, and Strobel, 2004; Atmosukarto et al., 2005).

A non-muscodor species (*Gliocladium* sp.) has also been discovered to produce volatile antibiotics. The volatile components of this organism are totally different than those of either *Muscodor albus* or *M. roseus*. In fact, the most abundant volatile inhibitor is [8]-annulene, formerly used as a rocket fuel and discovered here for the first time as a natural product. However, the bioactivity of the volatiles of this *Gliocladium* sp. is not as good or as comprehensive as that of the *Muscodor* spp. (Stinson, Ezra, and Strobel, 2003).

Antiviral Compounds from Endophytes

Another fascinating use of products from endophytic fungi is the inhibition of viruses. Two novel human cytomegalovirus (hCMV) protease inhibitors, cytonic acids A and B, have been isolated from solid-state fermentation of the endophytic fungus *Cytonaema* sp. Their structures were elucidated as *p*-tridepside isomers using MS and NMR methods (Guo et al., 2000). It is apparent that the potential for the discovery of compounds having antiviral activity from endophytes is in its infancy. The fact, however, that some compounds have been found already is promising. The main limitation to compound discovery to date is probably related to the absence of common antiviral screening systems in most compound discovery programs.

Endophytic Fungal Products as Anticancer Agents

Taxol and some of its derivatives represent the first major group of anticancer agents that are produced by endophytes (Figure 7). Taxol, a highly functionalized diterpenoid, is found in each of the world's yew (*Taxus*) species, but was originally isolated from *Taxus brevifolia* (Wani et al., 1971). The original target diseases for this compound were ovarian and breast cancers, but now it is used to treat a number of other human tissue–proliferating diseases. The presence of taxol in yew species prompted the study of their endophytes. By the early 1990s, however, no endophytic fungi had been isolated from any of the world's representative yew species. After several years of effort, a novel taxol-producing endophytic fungus, *Taxomyces andreanae*, was discovered in *Taxus brevifolia* (Strobel et al., 1993). The most critical line of evidence for the presence of taxol in the culture fluids of this fungus was the electrospray mass spectrum of the putative taxol isolated from *Taxomyces andreanae*. In electrospray mass spectrometry, taxol usually gives two peaks, one at m/z 854 $[M+H]^+$ and the other at m/z 876 $[M+Na]^+$. Fungal taxol had an identical mass spectrum to authentic taxol, and ^{14}C labeling studies showed the presence of fungal-derived taxol in the culture medium (Stierle,

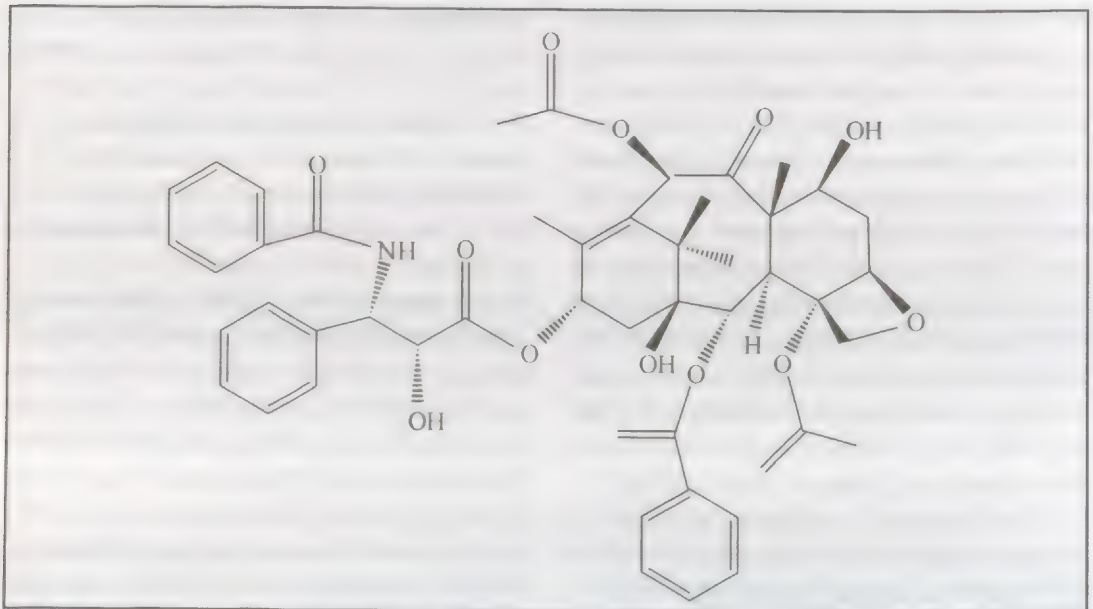

Figure 7. Taxol, the world's first billion-dollar anticancer drug, is produced by many endophytic fungi; it, too, possesses outstanding antioomycete activity.

Strobel, and Stierle, 1993). This early work set the stage for a more comprehensive examination of the ability of other *Taxus* species and many other plants to yield taxol-producing endophytes.

Some of the most commonly found endophytes of the world's yews, and many other plants, are *Pestalotiopsis* spp. (Strobel, 2002a, 2002b; Strobel and Daisy, 2003; Strobel et al., 2004). One of the most frequently isolated endophytic species is *P. microspora* (Strobel, 2002a). An examination of the endophytes of *Taxus wallichiana* Zucc. yielded *P. microspora*, and a preliminary monoclonal antibody test indicated that it might produce taxol (Walsh, 1992). After preparative thin-layer chromatography, a compound was isolated and shown by spectroscopic techniques to be taxol. Labeled (^{14}C) taxol was produced by this organism from several ^{14}C precursors that had been administered to it (Strobel et al., 1996).

Several other *Pestalotiopsis microspora* isolates were obtained from a bald cypress tree (*Taxodium distichum* (L.) Rich.) in South Carolina (U.S.) and were also shown to produce taxol (Li et al., 1996). This was the first indication that endophytes residing in plants other than *Taxus* spp. were producing taxol. Therefore, a specific search was conducted for taxol-producing endophytes on continents not known for any indigenous *Taxus* spp. This included investigating the prospects that taxol-producing endophytes exist in South America and Australia. In Australia, taxol-producing *Pestalotiopsis guepini* was isolated from the extremely rare, and previously thought to be extinct, Wollemi pine (*Wollemia nobilis* W.G. Jones, K.D. Hill & J.M. Allen) (Strobel et al., 1997). Also, quite surprisingly, a rubiaceous plant, *Maguireothamnus speciosus* (N.E. Br.) Steyerm., yielded a novel fungus, *Seimatoantlerium tepuiense*, which was shown to produce taxol. This endemic plant grows on the top of the tepuis on the Venezuela-Guyana border in southwest Venezuela (Strobel et al., 1999a). Taxol production has also been noted in *Periconia* sp. and the novel endophytic fungus *Seimatoantlerium nepalense* (Li et al., 1998; Bashyal et al., 1999).

Simply, it appears that the distribution of taxol-producing fungi is worldwide and is not confined to endophytes of yews. The ecological and physiological explanation for this seems to be related to the fact that taxol is a fungicide, and the most sensitive organisms to it are plant pathogens such as *Pythium* spp. and *Phytophthora* spp. (Young et al., 1992). These pythiaceous organisms are some of the world's most important plant pathogens and are strong competitors with endophytic fungi for niches within plants. In fact, their sensitivity to taxol is based on their interaction with tubulin in an identical manner as in rapidly dividing human cancer cells (Rowinsky, Cazenave, and Donehower, 1990; Young et al., 1992). Thus, bona fide endophytes may be producing taxol and related taxanes to protect their respective host plant from degradation and disease caused by these pathogens.

Other investigators have also made observations on taxol production by endophytes, including the discovery of taxol production by a *Tubercularia* sp. isolated from the Chinese yew (*Taxus mairei* (Lemée & H. Lév.) S.Y. Hu) in Fujian Province of southeastern mainland China (Wang et al., 2000). At least three endophytes of *Taxus wallichiana* produce taxol, including *Sporormia minima* and *Trichothecium* sp. (Shrestha et al., 2001). Using high-performance liquid chromatography and ionization mass spectrometry, taxol has been discovered in the hazelnut (filbert) tree (*Corylus avellana* L. cv. 'Gasaway') (Hoffman et al., 1998). Several fungal endophytes of this plant produce taxol in culture (Hoffman et al., 1998).

It is important to note, however, that taxol production by all endophytes in culture is in the range of sub-microgram to microgram per liter. Also, commonly, the fungi will attenuate taxol production in culture, increasing the possibility for recovery of the taxol, if certain activator compounds are added to the medium (Li et al., 1998). Efforts are being made to determine the feasibility of making microbial taxol a commercial possibility. The greatest prospect may be the

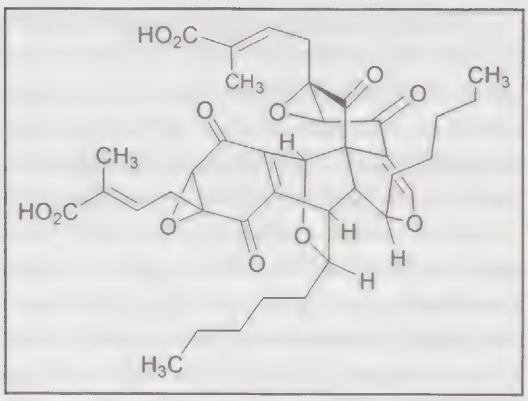

Figure 8. Torreyanic acid, an anticancer compound, from *Pestalotiopsis microspora.*

discovery of endophytes that make large quantities of one or more taxanes that could then be used as platforms for the organic synthesis of taxol or one of its anticancer relatives.

Torreyanic acid, a selectively cytotoxic quinone dimer and potential anticancer agent, was isolated from a *Pestalotiopsis microspora* strain (Figure 8). This strain was originally obtained as an endophyte associated with the endangered tree *Torreya taxifolia* Arn. (Florida Torreya) (Lee et al., 1996). Torreyanic acid was tested in several cancer cell lines; it demonstrated five to ten times more potent cytotoxicity in those lines that are sensitive to protein kinase C agonists and caused cell death by apoptosis. Recently, torreyanic acid has been successfully synthesized by application of a biomimetic oxidation/dimerization cascade (Li, Johnson, and Porco, 2003).

Alkaloids are also commonly found in endophytic fungi. The fungal genera *Xylaria*, *Phoma*, *Hypoxylon*, and *Chalara* are representative producers of a relatively large group of substances known as the cytochalasins, of which over 20 are now known. Many of these compounds possess antitumor and antibiotic activities but, because of their cellular toxicity, they have not been developed into pharmaceuticals. Three novel cytochalasins have recently been reported from *Rhinocladiella* sp. as an endophyte on *Tripterygium wilfordii*. These compounds have antitumor activity and have been identified as 22-oxa-[12]-cytochalasins (Wagenaar et al., 2000). Thus, it is not uncommon to find one or more cytochalasins in endophytic fungi. This provides an example of the fact that redundancy in discovery does occur, making dereplication an issue even for these under-investigated sources.

Endophytic Fungal Products as Antioxidants

Two compounds, pestacin and isopestacin, have been obtained from culture fluids of *Pestalotiopsis microspora*, an endophyte isolated from a combretaceous plant, *Terminalia morobensis* Coode, growing in the Sepik River drainage system of Papua New Guinea (Strobel et al., 2002; Harper et al., 2003). Both pestacin and isopestacin display antimicrobial as well as antioxidant activity. Isopestacin was attributed with antioxidant activity based on its structural similarity to the flavonoids (Figure 9). Electron spin resonance spectroscopy measurements confirmed this antioxidant activity, showing that the compound is able to scavenge superoxide and hydroxyl free radicals in solution (Strobel et al., 2002). Pestacin occurs naturally as a racemic mixture and also possesses potent antioxidant activity, at least one order of

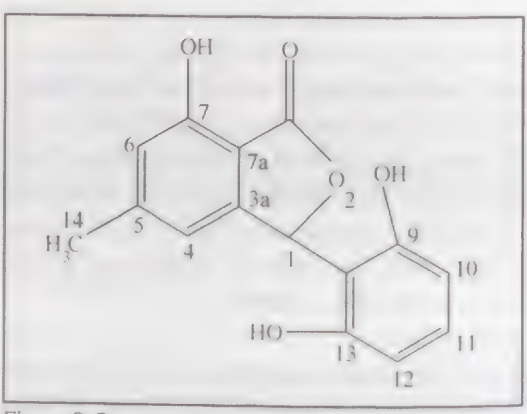

Figure 9. Isopestacin, an antioxidant produced by an endophytic *Pestalotiopsis microspora* strain, isolated from *Terminalia morobensis* growing on the north coast of Papua New Guinea.

magnitude more potent than that of Trolox, a vitamin E derivative (Harper et al., 2003). The antioxidant activity of pestacin arises primarily via cleavage of an unusually reactive C-H bond, and to a lesser extent, through O-H abstraction (Harper et al., 2003).

Endophytic Fungal Products as Immunosuppressive Compounds

Immunosuppressive drugs are used today to prevent allograft rejection in transplant patients, and in the future they could be used to treat autoimmune diseases such as rheumatoid arthritis and insulin-dependent diabetes. The endophytic fungus, *Fusarium subglutinans*, isolated from *Tripterygium wilfordii*, produces the immunosuppressive, but noncytotoxic diterpene pyrones, subglutinols A and B (Lee et al., 1995a). Subglutinols A and B are equipotent in the mixed lymphocyte reaction (MLR) assay and thymocyte proliferation (TP) assay with an IC_{50} of 0.1 μM. The famed immunosuppressant drug cyclosporin A, also a fungal metabolite, was roughly as potent in the MLR assay and 10^4 more potent in the TP assay. However, the lack of toxicity associated with subglutinols A and B suggests that they should be explored in greater detail as potential immunosuppressants (Lee et al., 1995a).

Surprising Results from Molecular Biological Studies on *Pestalotiopsis microspora*

Of some compelling interest is an explanation as to how the genes for taxol production may have been acquired by *Pestalotiopsis microspora* (Long et al., 1998). Although the complete answer to this question is not at hand, some other relevant genetic studies have been performed on this organism. *Pestalotiopsis microspora* Ne 32 is one of the most easily genetically transformable fungi that have been studied to date. In vivo addition of telomeric repeats to foreign DNA generates extra chromosomal DNA in this fungus (Long et al., 1998). Repeats of the telomeric sequence 5'-TTAGGG-3' were appended to non-telomeric-transforming DNA termini. The new DNA, carrying foreign genes and the telomeric repeats, replicated independently of the chromosome and expressed the information carried by the foreign genes. The addition of telomeric repeats to foreign DNA is unusual among fungi. This finding may have important implications in the biology of *P. microspora* Ne 32 since it explains at least one mechanism as to how new DNA can be captured by this organism and eventually expressed and replicated. Such a mechanism may begin to explain how the enormous biochemical variation may have arisen in this fungus (Li et al., 1996). Also, this initial work represents a framework to aid in the understanding of how this fungus may adapt itself to the environment of its plant hosts and suggests that the uptake of plant DNA into its own genome may occur. In addition, the telomeric repeats have the same sequence as human telomeres, which points to the possibility that *P. microspora* may serve as a means to make artificial human chromosomes, a totally unexpected result.

Concluding Statements

Endophytes are a poorly investigated group of microorganisms that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of applications. The mechanisms through which endophytes exist and respond to their surroundings must be better understood in order to be more predictive about which higher plants to seek, study, and employ in isolating their microfloral components. This may facilitate the natural product discovery process. This in turn may provide new platforms and leads for the pharmaceutical industry.

Although work on the utilization of this vast resource of poorly understood microorgan-

isms has just begun, it has already become obvious that an enormous potential for organism, product, and utilitarian discovery in this field holds exciting promise. This is evidenced by the discovery of a wide range of products and microorganisms that present potential as described in this chapter.

It is important for all involved in this work to realize the importance of acquiring the necessary permits from governmental, local, and other sources to collect and transport plant materials (especially from abroad) from which endophytes are to be eventually isolated. In addition to this aspect of the work is the added activity of producing the necessary agreements and financial-sharing arrangements with indigenous peoples or governments in case a product does develop an income stream.

Certainly, one of the major problems facing the future of endophyte biology and natural product discovery is the rapidly diminishing rainforests, which hold the greatest possible resource for acquiring novel microorganisms and their products. The total landmass of the world that currently supports rainforests is about equal to the area of the U.S. (Mittermeier, Myers, and Mittermeier, 2000). Each year, an area the size of Vermont or greater is lost to clearing, harvesting, fire, agricultural development, mining, or other human-oriented activities (Mittermeier, Myers, and Mittermeier, 2000). Presently, it is estimated that only a small fraction (10%–20%) of what were the original rainforests existing 1,000 to 2,000 years ago are currently present on the earth (Mittermeier, Myers, and Mittermeier, 2000). The advent of major negative pressures on them from these human-related activities appears to be eliminating entire mega-life forms at an alarming rate. Few have ever expressed information or opinions about what is happening to the potential loss of microbial diversity as entire plant species disappear. It can only be guessed that this microbial diversity loss is also happening, perhaps with the same frequency as the loss of mega-life forms, especially since certain microorganisms may have developed unique symbiotic relationships with their plant hosts. Thus, when a plant species disappears, so too does its entire suite of associated endophytes. Consequently, all of the capabilities that the endophytes might possess to provide natural products with medicinal potential are also lost. Multi-step processes are needed now to secure information and life forms before they are lost. Those places on earth that can be characterized as unique areas of biodiversity need immediate preservation. Countries need to establish information bases of their biodiversity and, at the same time, begin to make national collections of microorganisms that live in these areas. Endophytes are only one example of a life form source that holds enormous promise to impact many aspects of human existence. The problem of the loss of biodiversity should be one of concern to the entire world.

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The Metabolic Versatility of the Methymycin/Pikromycin Biosynthetic Pathway[1]

Jeffrey D. Kittendorf[2] and David H. Sherman[2*]

The methymycin/pikromycin (Pik) macrolide pathway represents a robust metabolic system for analysis of modular polyketide biosynthesis. Over the past decade, we have developed Pik to address key issues in the function, specificity, and catalytic properties of the six-module polyketide synthase, desosamine sugar assembly/glycosyltransfer, and the cytochrome P450 (CYP450) responsible for hydroxylation. Early studies relied on in vivo analysis to establish the precise function of individual components of the pathway and to provide key molecular genetic and protein biochemical tools for detailed studies of purified polyketide synthase (PKS) modules, and tailoring enzymes. This early work revealed that the Pik pathway evolved remarkable mechanisms for harnessing chemical diversity through the natural flexibility of the biosynthetic enzymes that yield six macrolide antibiotics from the combined activities of the native PKS and CYP450. These include ring-size variation of the macrolactone (12- vs. 14 membered rings), and multiple hydroxylation patterns on the macrolactone core. In addition, metabolic engineering of the sugar biosynthetic system provided access to new deoxysugars that were transferred to the macrolactone ring by a substrate-tolerant glycosyltransferase. Recent studies have focused on probing the molecular basis for macrolactone ring-size variation enabled by the Pik PKS docking domain interactions, and the unique ability of the CYP450 to modify multiple sites on the macrolactone ring. Investigation of ring-size variation has been pursued by a combination of in vitro studies using native and engineered Pik module 5/module 6 with natural pentaketide and hexaketide chain elongation intermediates as substrates, and x-ray crystallography with a range of affinity-labeled Pik TE catalytic domains. Moreover, x-ray crystallographic analysis and site-directed mutagenesis have revealed the basis for macrolide anchoring and selectivity in the PikC CYP450 leading to its diverse product profile. Targeted active site design has provided access to a novel PikC hydroxylase with enhanced functional properties paving the way for development of novel ketolide anti-infective agents.

Introduction

The rapid rise of antibiotic-resistant microbes has placed an ever-increasing demand on the discovery and development of novel antibacterial agents. Macrolide antibiotics, characterized by a glycoside-linked macrolactone ring, remain the most effective natural product anti-infective agents to date and continue to be featured in ongoing efforts toward the development of new therapeutics that target drug-resistant pathogens. Within this therapeutic class, the polyketide-derived natural product erythromycin was the first to be introduced into human use, and its structure continues to be a crucial template for the generation of semi-synthetic antibiotics, such as the ketolides. These compounds, which have recently been introduced into the clinic, have proven effective at combating previously resistant respiratory tract infections, as they do not induce the MLS_B family of resistance

[1]We thank the many past and present members of the Sherman Laboratory who have contributed to our current understanding of pikromycin biosynthesis. We also gratefully acknowledge the support and efforts of our many talented collaborators in this endeavor. The Sherman Laboratory is generously supported by grants from the National Institutes of Health (GM076477, CA108874, TW007404), the Hans and Ella McCollum Vahlteich Research Fund at the University of Michigan College of Pharmacy, and the H.W. Vahlteich Professorship in Medicinal Chemistry. JDK is supported by a National Research Service Award postdoctoral fellowship (GM075641) from the National Institutes of Health.

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(Nguyen and Chung, 2005). Given this success, the discovery of structurally diverse ketolides is highly desirable for combating the continuous emergence of multi-drug-resistant pathogens.

The structural complexity displayed by the macrolide class of antibiotics in general, and the ketolides in particular, presents substantial challenges for the synthesis of analog libraries of target compounds. Currently, ketolides are generated by a 12-step semi-synthesis starting with the 14-membered ring macrolide erythromycin A (Denis et al., 1999). To reduce this synthetic complexity, recent efforts have focused on harnessing the catalytic power and specificity of biosynthetic enzymes to increase the structural diversity of polyketide natural products, a technique often referred to as "combinatorial biosynthesis" (Kittendorf and Sherman, 2006).

The biosynthesis of macrolide polyketides relies on type I modular polyketide synthases (PKSs) to catalyze the step-wise condensation of simple carboxylic acid derivatives. Organizationally, type I PKSs are arranged into modules, wherein each module is comprised of a set of catalytic activities that is responsible for a single elongation of the polyketide chain and subsequent reductive processing of the β-keto functionality (Donadio and Katz, 1992). At a minimum, each elongation module contains an acyl transferase (AT) domain, an acyl-carrier protein (ACP) domain, and a ketosynthase (KS) domain. The AT domain is responsible for loading the appropriate CoA extender unit onto the ACP domain (i.e., malonyl-CoA, methylmalonyl-CoA, etc.). The KS domain then catalyzes a decarboxylative condensation of the extender unit with the growing polyketide chain obtained from the preceding module to generate an ACP-bound β-ketoacyl intermediate. In addition to the three core domains, each elongation module may contain up to three additional domains (ketoreductase [KR], dehydratase [DH], enoyl reductase [ER]) responsible for the reductive processing of the β-keto functionality prior to the next extension step. These reductive steps contribute greatly to the overall structural diversity that is observed among polyketide natural products. The presence of a KR domain alone gives rise to a β-hydroxyl functionality, the presence of both a KR and a DH domain generates an alkene, while the combination of KR, DH, and ER results in complete reduction to the alkane. Finally, a terminal thioesterase (TE) domain catalyzes the termination of polyketide biosynthesis. The activity of this domain results in the cleavage of the acyl chain from the adjacent ACP and the formation of the macrolactone ring via intramolecular cyclization. Additional tailoring enzymes, such as hydroxylases and glycosyl transferases, serve to further modify the macrolactone aglycones. These tailoring steps are often critical for bioactivity of the resultant compounds.

PIKROMYCIN AND THE PIKROMYCIN PKS: PATHWAY CHARACTERIZATION AND METABOLIC DIVERSITY

A modular type I PKS system in *Streptomyces venezuelae* is known to produce the naturally occurring ketolide antibiotic, pikromycin. While not clinically useful as an anti-infective agent, pikromycin offers a structural template for the semisynthetic generation of ketolide analogs. Moreover, exploitation of the biosynthetic enzymes responsible for the synthesis of pikromycin offers an attractive biosynthetic strategy for generating a diverse library of ketolide compounds. To this end, our laboratory has been engaged in a highly cross-disciplinary and collaborative research program aimed at developing a detailed understanding of pikromycin biosynthesis and leveraging the pikromycin biosynthetic enzymes for the production of novel macrolide antibiotics.

Although pikromycin was first isolated in 1951 (Brockmann and Henkel, 1951), the origin of its biosynthesis was not elucidated until almost five decades later, when Xue et al. reported the cloning and subsequent sequence of the pikromycin biosynthetic gene cluster from *Strepto-*

myces venezuelae ATCC 15439 (Figure 1) (Xue et al., 1998b). DNA sequence analysis identified 18 discrete genes that could readily be divided into five separate loci: PKS (*pikA*), desosamine biosynthesis (*des*), cytochrome P450 hydroxylase (*pikC*), a transcriptional activator (*pikD*), and a resistance locus (*pikR*). While the Sherman Laboratory remains devoutly interested in all aspects of pikromycin biosynthesis, a majority of work over the last several years has been directed at investigating and exploiting the biosynthetic enzymes encoded by *pikA*, *des*, and *pikC*.

The *pikA* gene locus encodes five polypeptides (PikAI–PikAV) that together encompass a type I modular polyketide synthase (Pik PKS) (Figures 1 and 2). The first four polypeptides, PikAI–PikAIV, are composed of a single loading module and six PKS elongation modules that are

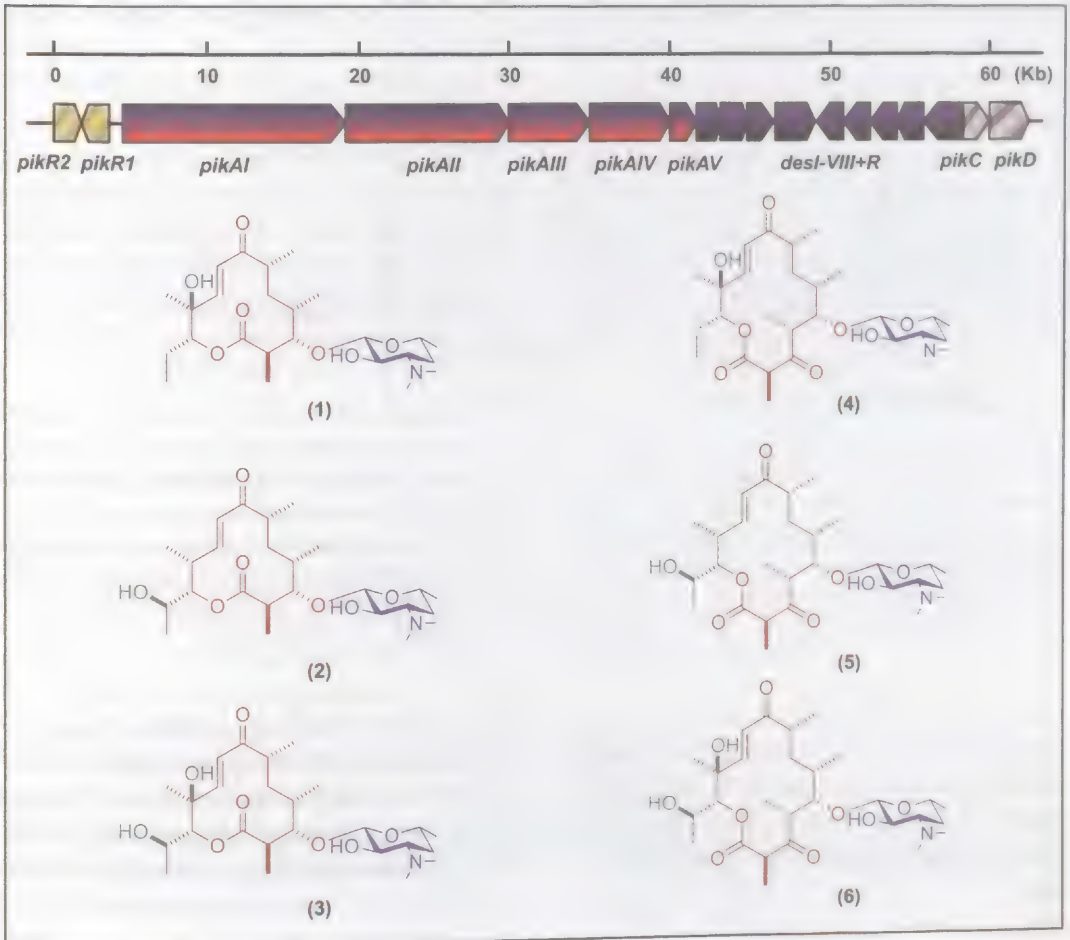

Figure 1. The methymycin/pikromycin biosynthetic pathway. The methymycin/pikromycin biosynthetic pathway is comprised of 18 discrete genes, clustered into five separate loci, which are responsible for the generation of six different macrolide antibiotics. The *pikA* locus encodes a type I PKS that directs the biosynthesis of both the 12- and 14-membered macrolactone rings (shown in red). The *des* locus encodes all proteins responsible for the generation of the desosamine sugar moiety and attachment to the macrolactone core (shown in blue). *PikC* is a cytochrome P450 that serves to hydroxylate the C12 and/or C14 positions of the macrolides (shown in grey). Compounds: (1) methymycin, (2) neomethymycin, (3) novamethymycin, (4) pikromycin, (5) neopikromycin, (6) novapikromycin.

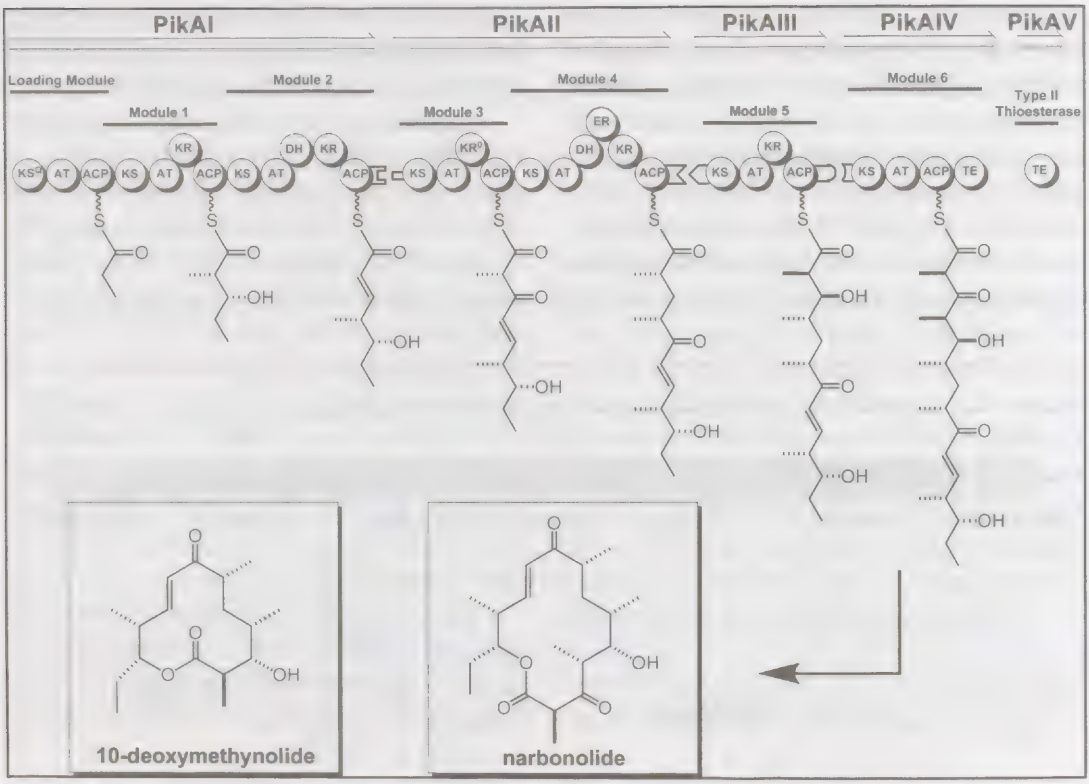

Figure 2. The pikromycin type I polyketide synthase. The pikromycin polyketide synthase contains one loading module and six elongation modules spanning four polypeptide chains (PikAI–PikAIV). Chain elongation through PikAIII followed by thioesterase catalyzed termination results in the 12-membered macrolactone product, 10-deoxymethynolide, while continued elongation of the polyketide chain produces the 14-membered macrolactone narbonolide. Both products undergo further processing via a glycosyl transferase and P450 hydroxylase to yield the methymycin and pikromycin suite of antibiotics. Abbreviations: KS, ketosynthase; AT, acyl transferase; ACP, acyl carrier protein; KR, ketoreductase; DH, dehydratase; ER, enoyl reductase; TE, thioesterase.

responsible for directing macrolactone biosynthesis. Notably, the final two elongation modules are monomodular, as each are contained on separate polypeptides (PikAIII and PikAIV). PikAV is a type II thioesterase and has been shown to serve a critical editing role, functioning to remove aberrantly decarboxylated extender units from PKS ACP domains (Kim et al., 2002).

Shortly after completing the sequence analysis of the *pikA* locus, genetic disruption studies were commenced and the results unambiguously demonstrated that the pikromycin PKS is unique from other known PKS systems, as it is capable of efficiently generating two distinct macrolactone products (Xue et al., 1998b). Premature termination of polyketide biosynthesis at PikAIII results in the production of the 12-membered ring macrolactone, 10-deoxymethynolide, while continued elongation through PikAIV, followed by TE catalyzed cyclization results in the synthesis of the 14-membered macrolactone narbonolide (Figure 2). Remarkably, both 10-deoxymethynolide and narbonolide undergo further tailoring via the *desVII*-encoded glycosyl transferase (Zhao, Sherman, and Liu, 1998) and the *pikC*-encoded hydroxylase (Xue et al., 1998a) to yield a suite of antibiotic compounds that consists of the 12-membered ring macrolides,

methymycin, novamethymycin, and neomethymycin, as well as the 14-membered ring macrolides pikromycin and narbomycin. Together, the unique biosynthetic capabilities of the pikromycin PKS, coupled with the substrate flexibility of the respective tailoring enzymes, position the pikromycin biosynthetic system as a potentially powerful tool for the generation of novel macrolide antibiotic compounds.

SUGAR PATHWAY ENGINEERING

The earliest attempts to leverage the pikromycin biosynthetic system for the production of novel compounds involved manipulation of the genes required for the biosynthesis of the desosamine. The initial sequencing of the pikromycin biosynthetic pathway indicated that the *des* locus contains the full complement of genes required for the biosynthesis of this amino sugar (Figure 1) (Xue et al., 1998b), while genetic knockout experiments illuminated the precise sequence of biosynthetic events that give rise to its production (Zhao, Sherman, and Liu, 1998; Borisova et al., 1999). Interestingly, several of these gene knockouts produced novel macrolides that contained desosamine intermediates. For example, inactivation of *desI* resulted in the replacement of desosamine with *D*-quivinose, inactivation of *desV* resulted in the incorporation of 4,6-dideoxyhexose, while inactivation of *desVI* resulted in the incorporation of 3-*N*-acylated amino sugars. To achieve further structural diversity, the 4-aminotransferase from the calicheamicin biosynthetic pathway (*calH*) was introduced into the *desI* knockout (Zhao et al., 1999). Encouragingly, this complementation experiment successfully produced novel compounds containing the predicted 4-amino sugars. Most significantly, this work, along with the knockout experiments described above, demonstrated that the *desVII*-encoded glycosyl transferase is not only able to accommodate macrolactone substrates of different sizes (12- and 14-membered ring macrolactones) but can also bind and incorporate a variety of unnatural sugars. Given this substrate flexibility, this single glycosyl transferase appears to be well suited for use in future combinatorial biosynthetic strategies aimed at the production of novel macrolide compounds.

INVESTIGATIONS OF PIKC: UNDERSTANDING HYDROXYLATION

The *pikC*-encoded cytochrome P450 hydroxylase lends itself as yet another tool for the generation of structurally diverse macrolide antibiotic compounds. PikC, the sole P450 hydroxylase present in the pikromycin biosynthetic gene cluster (Xue et al., 1998b), displays unparalleled flexibility toward the macrolactone core of its substrates (Figure 1) (Xue et al., 1998a). This hydroxylase is capable of mono-hydroxylating at either the C12 or the C14 positions of the 12-membered ring macrolactone YC-17, giving rise to methymycin and neomethymycin, respectively. PikC can also dihydroxylate at both the C12 and C14 positions to generate novamethymycin. With respect to 14-membered macrolactone substrates, PikC-catalyzed hydroxylation of the C12 position of narbomycin gives rise to the macrolide antibiotic, pikromycin, while hydroxylation at C14 results in the production of neopikromycin (Lee et al., 2006). Additionally, it has been demonstrated that PikC can also catalyze dihydroxylation of narbomycin to give novapikromycin (Lee et al., 2006).

The diverse hydroxylation patterns that can be achieved via PikC catalysis raise an important question regarding substrate recognition and binding. How is it that a single enzyme can not only recognize macrolactones of different sizes but also hydroxylate at different positions around each macrolactone ring? The answer to these questions was found in the recently reported x-ray co-crystal structures of PikC, bound with either the 12-membered macrolide YC-17 or the 14-membered macrolide narbomycin (Sherman et al., 2006). Interestingly, the 12- and 14-mem-

bered ring macrolactone portions of both macrolide substrates bind in the active site utilizing the same set of protein-substrate contacts; however, the desosamine moiety can occupy one of two different binding pockets, depending on the size of the macrolactone substrate. Within each desosamine binding pocket, the most critical interaction involves the sandwiching of the C3' dimethylamino group by a pair of carboxylate-containing residues (Glu and Asp). Undoubtedly, this enhanced insight into PikC substrate binding provides a tremendous opportunity to rationally design unnatural glycosylated substrates for PikC hydroxylation. Moreover, it enables the facile selection of one of the two sugar-binding pockets, via site-directed mutagenesis, in order to shift reaction product distribution to a single bioactive product.

PikAIII and PikAIV: An In Vitro Understanding of Chain Elongation

Recent work has also focused on in vitro experiments that are aimed at investigating the chain elongation and termination events catalyzed by the final two elongation monomodules of the pikromycin PKS (PikAIII and PikAIV) (Figure 2). To this end, each monomodular protein was co-expressed in *Escherichia coli* BL21(DE3) with the phosphopantetheinyl transferase from *Bacillus subtilis* (*sfp*) (Beck et al., 2003a). Co-expression with this promiscuous phosphopantetheinyl transferase was performed to ensure the efficient phosphopantetheinyl modification of the ACP domains that reside within the individual multifunctional proteins. Subsequently, a series of diketide *N*-acetylcysteamine (NAC) thioesters was synthesized and used to investigate initiation, extension, skipping, and termination leading to triketide and tetraketide lactone structures from PikAIII and PikAIII-PikAIV (Beck et al., 2003a, 2003b). Notably, this work provided the first comparable analysis to the 6-deoxyerythronolide B (DEBS) PKS system. An important discovery made during these initial in vitro studies was that the non-natural diketide substrates, routinely used during the in vitro characterizations of the DEBS PKS, do not adequately substitute for the natural polyketide substrates of the pikromycin PKS system, as the measured k_{cat} values were approximately three orders of magnitude lower than the in vivo rates of pikromycin production that are observed in *Streptomyces venezuelae*.

To more accurately mimic the naturally occurring chain elongation reaction that is catalyzed by the combination of PikAIII and PikAIV, the synthesis of the natural-length pentaketide substrate for PikAIII was undertaken and successfully accomplished (Aldrich et al., 2005a). Remarkably, kinetic analysis with this substrate yielded an apparent k_{cat} that is approximately 50-fold higher than that determined with the diketide substrates. Furthermore, the apparent K_M value for the pentaketide was approximately 25-fold lower than that of the best diketide substrate. Thus, the specificity constant (as defined by k_{cat}/K_M) is over 1,500-fold greater for the natural pentaketide substrate versus the unnatural diketide analogs. These results strongly suggest that the use of natural chain-length polyketide intermediates is crucial for obtaining a full mechanistic understanding of these multifunctional proteins.

Together, the ability to produce recombinant PikAIII and PikAIV, coupled with the ready availability of the pentaketide chain elongation intermediate, offers the tremendous opportunity to perform detailed interrogations into the mechanism that drives the formation of multiple macrolactone products. Currently, our efforts in this area are focused on exploring the role of the docking domain–mediated protein-protein interaction between PikAIII and PikAIV. Preliminary results suggest that this protein-protein interaction is required for the production of 10-deoxymethynolide (Kittendorf et al., 2007). Furthermore, we have demonstrated that PikAIII, when paired with Pik TE in *trans*, can extend

the synthetic pentaketide substrate to generate the 12-membered macrolactone (Kittendorf et al., 2007). While this work remains ongoing, it is anticipated that these investigations may lead to the ability to control the ratio of 12- and 14-membered ring production in the pikromycin PKS system, allowing for a shift in the product distribution that favors a desired ring size. Additionally, enhanced insight into this unique phenomenon may enable this mechanism to be incorporated into a variety of PKS systems, thereby increasing the potential pool of macrolactone compounds that could be generated via manipulation of PKS biosynthetic pathways.

MACROLACTONE CYCLIZATION

In vitro experiments have also investigated the macrolactone cyclization by the terminal thioesterase domain (Pik TE) of the pikromycin PKS system. It has previously been established that this single thioesterase domain is responsible for cyclizing both macrolactone compounds that are produced by the Pik PKS (Xue and Sherman, 2000). Given this, a detailed investigation into reaction kinetics, substrate tolerance, and catalytic mechanism could lead to the transformation of this enzymatic domain into a general macrolactonization catalyst.

As the cloning, expression, and purification of the recombinant pikromycin thioesterase were previously established (Lu et al., 2002), work in this area began with the synthesis of full-length substrates that could be cyclized by the thioesterase. The hexaketide substrate, which is the precursor to 10-deoxymethynolide, was successfully generated by both total synthetic (Aldrich et al., 2005a) and semisynthetic strategies (Aldrich et al., 2005b). In vitro, Pik TE demonstrated the ability to catalyze the complete conversion of this linear polyketide intermediate to the 12-membered ring macrolactone. Poor aqueous solubility of the substrate prevented the determination of kinetic parameters; however, the specificity constant (k_{cat}/K_M) was estimated to be 1.7 mM^{-1} min^{-1} (Aldrich et al., 2005b). Interestingly, preliminary substrate specificity investigations revealed that the enzyme was sensitive to structural changes at the C7 position of the hexaketide substrate (Aldrich et al., 2005b). The native substrate, containing a carbonyl at the C7 position, is exclusively cyclized by the enzyme; however, reduction of the carbonyl to the alcohol results in a hexaketide substrate that gets exclusively hydrolyzed to the free acid. This interesting finding suggests that, despite its natural substrate tolerance for chain length variation, Pik TE is sensitive to minor functional group modifications of its natural substrates.

To further examine the substrate specificity requirements of this enzyme, we sought to gain enhanced insight into the active site architecture of this catalytic domain via x-ray crystallography. To this end, Fecik and co-workers have demonstrated that diphenylphosphonate triketides can effectively serve as Pik TE affinity labels, offering a valuable tool for the examination of the substrate specificity and catalytic mechanism of this PKS domain (Giraldes et al., 2006). Co-crystallization studies with these compounds revealed the presence of a non-classical oxyanion hole in which only a single hydrogen bond is donated from an active site residue (Giraldes et al., 2006). It is hypothesized that the absence of a second hydrogen bond donor is due to the need to accommodate both ends of the polyketide during cyclization of the acyl-serine intermediate. As a follow-up, recent structural studies with an extended chain diphenylphosphonate heptaketide mimic have yielded remarkable insight into Pik TE catalysis (Akey et al., 2006). Specifically, this structure indicates that in the absence of direct protein-substrate interactions, substrate cyclization is directed via a hydrophilic barrier in which several well-ordered water molecules serve to reroute the hydrophobic substrate back toward the active site. Additionally, modeling studies suggest that the presence of an α/β unsaturated ketone

serves to constrain the conformation of the substrate, effectively directing the terminal hydroxyl for direct attack of the acyl-intermediate. While it remains to be determined if Pik TE could be developed into a general macrolactonization catalyst, work remains ongoing to better understand and expand upon the substrate tolerance of this PKS catalytic domain.

SUMMARY

The manipulation of modular PKS systems offers an exciting strategy for the generation of structurally diverse libraries of novel natural products that have potential medicinal value (Kittendorf and Sherman, 2006). However, due to limited understanding of PKS molecular mechanisms, catalytic activities, kinetic parameters, and protein-protein interactions, this potential remains largely unrealized. Over the last decade, our laboratory has been pioneering an understanding of these issues through the focused investigation of the pikromycin PKS from *Streptomyces venezuelae*. Through this work, we have gained fundamental insight into all stages of pikromycin biosynthesis. Undoubtedly, this knowledge will serve as the foundation for continued efforts aimed at leveraging the catalytic power and specificity of the pikromycin biosynthetic system for the production of novel ketolide anti-infective compounds.

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Realizing Nature's Potential: The Once and Future King of Drug Discovery was a two-day event held at the Missouri Botanical Garden in the fall of 2006 in honor of Dr. Gordon Cragg. Dr. Cragg, former chief of the Natural Products Branch of the National Cancer Institute, was throughout his career a tireless advocate for the conservation of nature in order to maintain the rich chemical diversity that has so often been vital to the development of new pharmaceuticals.

Research scientists from academic and corporate programs came together to present the results of their research in the natural products field. Their contributions touch upon recent methodological innovations, the legal and ethical landscape, and the future direction of natural products research.

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ISBN 978-1-930723-96-2

Cover photo: ©Gregory Basco/www.deepgreenphotography.com